

Novel Salmonella vectors with *in vivo* amplifiable plasmids for vaccination and tumor therapy

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Für Gretchen

Table of contents

1	Introduction.....	1
1.1	Vaccination and immunotherapy.....	1
1.1.1	Requirements of an effective vaccine.....	2
1.1.2	Types of vaccines	3
1.1.3	Novel vaccination approaches.....	4
1.1.3.1	Genetic immunization.....	4
1.1.3.2	Live viral and bacterial carriers.....	6
1.1.3.3	Routes of immunization	6
1.1.3.4	Sequential immunization.....	7
1.1.4	Immunotherapy	8
1.1.4.1	Therapeutic vaccines for chronic infections.....	8
1.1.4.2	Therapeutic vaccines for cancer therapy	9
1.2	Bacteria as delivery systems for prophylactic and therapeutic molecules.....	10
1.2.1	Bacteria as antigen delivery systems	10
1.2.1.1	Bacteria-mediated delivery of heterologous antigens	11
1.2.1.2	Bacteria-mediated delivery of DNA	12
1.2.2	Bacteria in cancer treatment	15
1.3	Salmonella as vectors for vaccination and tumor therapy	17
1.3.1	The course of a Salmonella infection	17
1.3.2	Attenuation of Salmonella for the use as live vector	21
1.3.3	Salmonella as antigen delivery system.....	22
1.3.3.1	Salmonella-mediated delivery of heterologous antigens.....	23
1.3.3.2	Salmonella-mediated delivery of plasmid DNA	24
1.3.4	Salmonella in cancer treatment.....	27
1.4	Improvement of Salmonella as delivery system in vaccination and tumor therapy	28
1.4.1	Attenuation of the bacterial carrier.....	28
1.4.2	Stability of the recombinant phenotype	29
1.4.3	Targeting of antigen delivery	31
1.4.4	Lysis of the bacterial carrier	32
1.4.5	Multivalent Salmonella vaccines.....	33
1.4.6	Regulatory elements	33
1.4.6.1	Choice of promoter	33
1.4.6.2	Remote control of bacterial carriers	35
1.5	Aims of this work.....	36
2	Material and methods.....	38
2.1	Bacterial strains and growth conditions.....	38
2.2	Cell lines.....	38
2.3	Mouse strains.....	38
2.4	Oligonucleotides.....	39
2.5	Plasmids	39
2.5.1	Outgoing plasmids for cloning	39
2.5.2	Reporter plasmids for L-arabinose inducible amplification	41
2.5.3	Reporter plasmids for <i>in vivo</i> inducible plasmid amplification	43
2.5.4	Plasmids for Tn7-mediated transposition	45
2.5.5	Expression plasmids with listeriolysin O as antigen	46
2.6	Chromosomal integration derivatives of SL7207.....	46
2.7	Molecular biological methods.....	48
2.7.1	Agarose gel electrophoresis.....	48
2.7.2	Preparation of DNA.....	48
2.7.2.1	Isolation of DNA from agarose gels	48
2.7.2.2	Isolation of plasmid DNA	48
2.7.2.3	Determination of DNA concentration	48
2.7.3	Molecular cloning.....	49
2.7.4	Polymerase chain reaction.....	49
2.8	Microbiological methods.....	50
2.8.1	Long-term storage of bacteria	50

2.8.2	Transformation of chemically competent bacteria	51
2.8.3	Transformation of electrocompetent bacteria	51
2.8.4	Preparation and transformation of chemically competent DH5 α pir	51
2.8.5	Determination of reporter gene expression and plasmid amplification <i>in vitro</i>	52
2.8.6	Tn7-mediated transposition in <i>Salmonella</i>	53
2.8.7	Determination of plasmid stability <i>in vitro</i>	53
2.8.8	Flow cytometry of bacteria	54
2.8.8.1	Flow cytometry of bacterial cultures	54
2.8.8.2	Flow cytometry of bacteria from infected cells and mice	54
2.8.8.3	Live/dead staining of bacterial cultures for flow cytometry	54
2.9	Tissue culture	55
2.9.1	Determination of viable cells	55
2.9.2	Detachment of adherent cells	55
2.9.3	Cryoconservation and thawing of cells	55
2.9.4	CFSE labeling	55
2.10	Biochemical methods	56
2.10.1	SDS-PAGE	56
2.10.2	Western Blot	56
2.10.3	Determination of L-arabinose concentration in blood plasma	57
2.11	Immunological methods	57
2.11.1	Flow cytometry	57
2.11.2	Antibodies for flow cytometry	58
2.11.3	Magnetic cells sorting	58
2.12	Determination of reporter gene expression in mouse macrophages	58
2.12.1	Infection of macrophages with recombinant <i>Salmonella</i>	58
2.12.2	Preparation of infected mouse macrophages for flow cytometry	59
2.12.3	Preparation of infected mouse macrophages for luciferase assay	59
2.13	Immunization with recombinant <i>Salmonella</i>	59
2.13.1	Infection of mice	59
2.13.2	Colonization and plasmid stability <i>in vivo</i>	60
2.13.3	Preparation of infected mouse organs for flow cytometry	60
2.13.4	Adoptive transfer of DO11.10 T cells and immunization with Ova ₃₂₃₋₃₃₉ expressing <i>Salmonella</i>	60
2.13.5	<i>In vivo</i> cytotoxicity assay for LLO ₉₁₋₉₉ specific immune responses	61
2.13.5.1	Immunization of mice	61
2.13.5.2	<i>In vivo</i> cytotoxicity assay	61
2.14	Infection of tumor-bearing mice	62
2.15	Non-invasive <i>in vivo</i> imaging	63
3	Results	64
3.1	Establishment of an <i>in vivo</i> inducible plasmid amplification system	64
3.1.1	Characterization of <i>in vivo</i> inducible promoters in <i>S. typhimurium</i> SL7207	64
3.1.2	Construction of strains with inducible plasmid amplification	66
3.1.3	Simultaneous amplification of plasmid replication and antigen expression	69
3.1.4	Colonization of mice by strains with inducible plasmid amplification	73
3.1.5	Immunization of mice	77
3.1.5.1	Generation of CD4 ⁺ T cell responses	77
3.1.5.2	Generation of cytotoxic T cells	80
3.1.6	Simultaneous <i>in vivo</i> inducible plasmid amplification and remote control of bacterial lysis by L-arabinose	83
3.1.7	Non-invasive <i>in vivo</i> imaging of P _{<i>slfA</i>} induction	85
3.2	Remote control of plasmid amplification by L-arabinose	88
3.2.1	Amplification of a single-copy plasmid controlled by plasmid-encoded P _{<i>BAD</i>}	88
3.2.2	Amplification of a single-copy plasmid controlled by chromosomally integrated P _{<i>BAD</i>}	91
3.2.3	Remote control of simultaneous amplification of plasmid replication and protein expression	93
3.2.4	Remote control of plasmid amplification in tumor-colonizing SL7207	95

4	Discussion.....	101
4.1	Establishment of an <i>in vivo</i> inducible plasmid amplification system.....	101
4.2	Remote control of plasmid amplification by L-arabinose	107
4.3	Concluding remarks	110
5	Summary.....	112
6	Abbreviations.....	114
7	References.....	117

1 Introduction

One of mankind's greatest achievements has been the development of vaccines to prevent infectious diseases. The introduction of vaccination by Jenner more than two hundred years ago and the discovery of chemotherapeutics by Ehrlich and antibiotics by Fleming a century later contributed to the decline of diseases that had been responsible for most of human mortality and morbidity during history. Now, vaccination is considered the most cost-effective and efficacious medical intervention against pathogens (Rappuoli et al. 2002).

1.1 Vaccination and immunotherapy

The term “vaccine” was first coined by Edward Jenner in 1796 after his discovery that prior exposure to cowpox virus prevents infection by the smallpox virus. Louis Pasteur later extended the concept of inducing a protective immune response to other pathogens (Silverstein 1999). Today, many bacterial and viral diseases like diphtheria, tetanus, measles and yellow fever can be prevented by vaccination. As proposed by Jenner, his new vaccination procedure could be used to eliminate smallpox. Indeed, the global eradication of smallpox was declared in 1980 (Fenner et al. 1988). Due to the immunization programs of the World Health Organization, poliomyelitis is expected to be eradicated in the near future.

Despite this success, a number of pathogens can not be controlled by current vaccination approaches. These pathogens include the three major killers HIV, *Mycobacterium tuberculosis* and the malaria parasite *Plasmodium falciparum* (Fig. 1.1). Consequently, about 11 million of 57 million annual deaths worldwide are estimated to be related directly to infectious diseases. The re-emergence of old diseases, as well as newly emerging diseases like SARS greatly magnify the global burden of infections (Morens et al. 2004). Microbial adaptation and evolution, e.g. the occurrence of drug-resistant microbes due to the vast use of antibiotics in veterinary and human medicine, in addition to international travel and inconsiderate human behavior are factors that contribute to the challenges of infectious diseases to public health. To meet these challenges, there is a need to continuously develop novel vaccines, as well as to improve existing vaccines in terms of safety and efficacy. There is also increasing interest in designing vaccines for so called

immunotherapeutic approaches that may be effective as therapeutic measure for chronic established infections but also for cancer and other diseases.

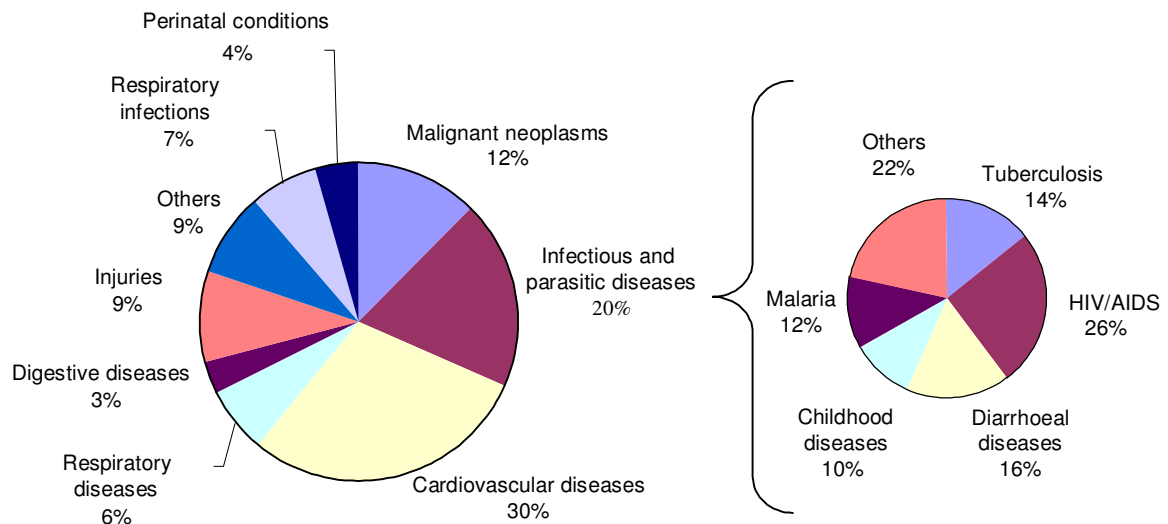


Fig. 1.1: Leading causes of death worldwide, estimates for 2002 (WHO 2004).

1.1.1 Requirements of an effective vaccine

The requirements of an effective vaccine depend on the nature of the infectious agent and the person to be immunized. Protective immunity against most viruses as well as against bacteria and parasites replicating extracellularly is mediated by humoral immune responses. In contrast, some bacteria such as *Mycobacterium tuberculosis* are obligate intracellular organisms, as they only replicate inside a susceptible cell. To control such infections, the induction of an effective cell mediated immune response, particularly of cytotoxic T cells and T-helper-1 (Th1) CD4⁺ cells, is essential (Ada 2005). For some viral infections like human immunodeficiency virus (HIV) both humoral and cellular response are likely to be required (Lima et al. 2004). Thus, a successful vaccine should elicit a suitable type of immunity. Furthermore, an effective vaccine has to induce protective immunity preferably in a high proportion of the population. In addition, it is desirable to induce lifelong immunological memory via a single administration of the vaccine, as it is impractical to administer booster immunizations to large populations especially in developing countries. Moreover, practical and safety issues have to be considered in designing vaccines. This

includes low costs, biological stability (e.g. no need for a cold chain), ease of administration and not least few side effects (Babiuk 1999).

1.1.2 Types of vaccines

The principle of vaccination is to mimic a natural infection in order to induce a long lasting immune response such that subsequent exposures to the infectious agent do not cause disease. One conventional way is to administer whole-cell vaccines that consist either of killed or live, attenuated microorganisms. Attenuated strains can be made by repeated passage of the infectious agent in tissue culture or animal host until loss of virulence and retention of immunogenicity are properly balanced (Ada 2001). Today, attenuation can be achieved more reliably by employing recombinant DNA techniques. Examples for live, attenuated viruses include the measles, mumps and rubella vaccines. Although live vaccines are often effective in stimulating strong immune responses, their application is limited due to safety concerns regarding the risk of virulence reversion. In contrast, inactivated whole microorganisms are non-infectious but also less immunogenic especially in terms of cell-mediated immunity and therefore often require booster injections and adjuvants (Ulmer et al. 2006). However, killed vaccines are useful for antibody-mediated prevention of viral diseases such as influenza.

Knowledge of microbial pathogenesis, identification of major virulence factors and characterization of the immune response after infection have led to the development of subunit vaccines based on purified antigenic components such as toxoids, bacterial polysaccharides and viral surface proteins (Scarselli et al. 2005). Effective vaccines against *Clostridium tetani* and *Corynebacterium diphtheriae* are based on the generation of neutralizing antibodies against detoxified versions of their secreted toxins. Similarly, antibodies, which protect via opsonization, can be induced against polysaccharides extracted from cultures of capsular bacteria.

Application of modern molecular biology techniques has resulted in the ability to clone, purify and engineer subunit antigens to remove toxic effects while retaining immunogenicity. These techniques gave rise to the first recombinant subunit vaccine against the hepatitis B virus (HBV), whose surface antigen is produced in recombinant yeast (McAleer et al. 1992).

Purified antigens are often administered with adjuvants to enhance their immunogenicity (Marciani 2003). Adjuvants represent products that increase or modulate the humoral or cellular immune response against an antigen. They can act via immunomodulation, induction of cytotoxic T lymphocytes (CTL), targeting of specific cells and depot generation (Cox and Coulter 1997). Adjuvants may for example provide “danger signals” which are recognized by pattern recognition receptors present on cells of the innate immune system thereby activating them (Medzhitov and Janeway, Jr. 2002). Alum (aluminum potassium phosphate), the adjuvant most often used, delays the release of an antigen such as the Hepatitis B vaccine at the injection site and therefore creates a depot (Lima et al. 2004).

1.1.3 Novel vaccination approaches

To date, most licensed vaccines generate protective antibodies that directly attack microorganisms and subsequently prevent infection. The many remaining challenges, such as vaccines against HIV, malaria, chronic infections and cancer, may require the induction of cellular immune responses. To achieve these types of responses, new approaches in vaccine development have to be made concerning the way vaccine antigens are presented, delivered and administered (Ulmer et al. 2006).

1.1.3.1 Genetic immunization

Genetic immunization is the use of DNA encoded antigens for vaccination. First experiments have shown that the direct injection of eukaryotic expression plasmids into mouse muscle lead to gene expression *in vivo* (Wolff et al. 1990) and to immune responses against the expressed protein (Tang et al. 1992). Since then, the induction of cell-mediated immune responses and protective antibodies was described in a number of animal models against viral, bacterial and parasitic diseases (Donnelly et al. 1997). The DNA can be injected either directly into muscles or as DNA-coated gold beads using a gene gun. After injection, antigen-encoding plasmids are taken up either by muscle or skin cells, or directly by antigen-presenting cells (APC) and remain episomally in these cells (Fig. 1.2) (Ertl and Xiang 1996). Subsequently, the gene is expressed. The resulting protein is processed similarly to a viral antigen and presented bound to MHC class I molecules. This leads to activation of CTL (Ulmer et al. 1993). Transfer of the antigen to APC can also occur via

cross-presentation (Stevenson 2004). Thus, T helper cells and B cells can be stimulated (Davis et al. 1993).

Advantages of genetic immunization include low costs of the plasmid DNA, its stability and the absence of infectious properties (Leitner et al. 1999). The plasmid DNA can be produced in *E. coli* using standard fermentation and downstream processing techniques that yield a highly stable and pure vaccine (Schleef and Schmidt 2004). Moreover, bacterial plasmids possess unmethylated CpG motifs that can function as adjuvant (Krieg 2000). These CpG motifs are recognized by Toll-like receptor (TLR) 9 and support a strong Th1-like inflammatory response (Hemmi et al. 2000).

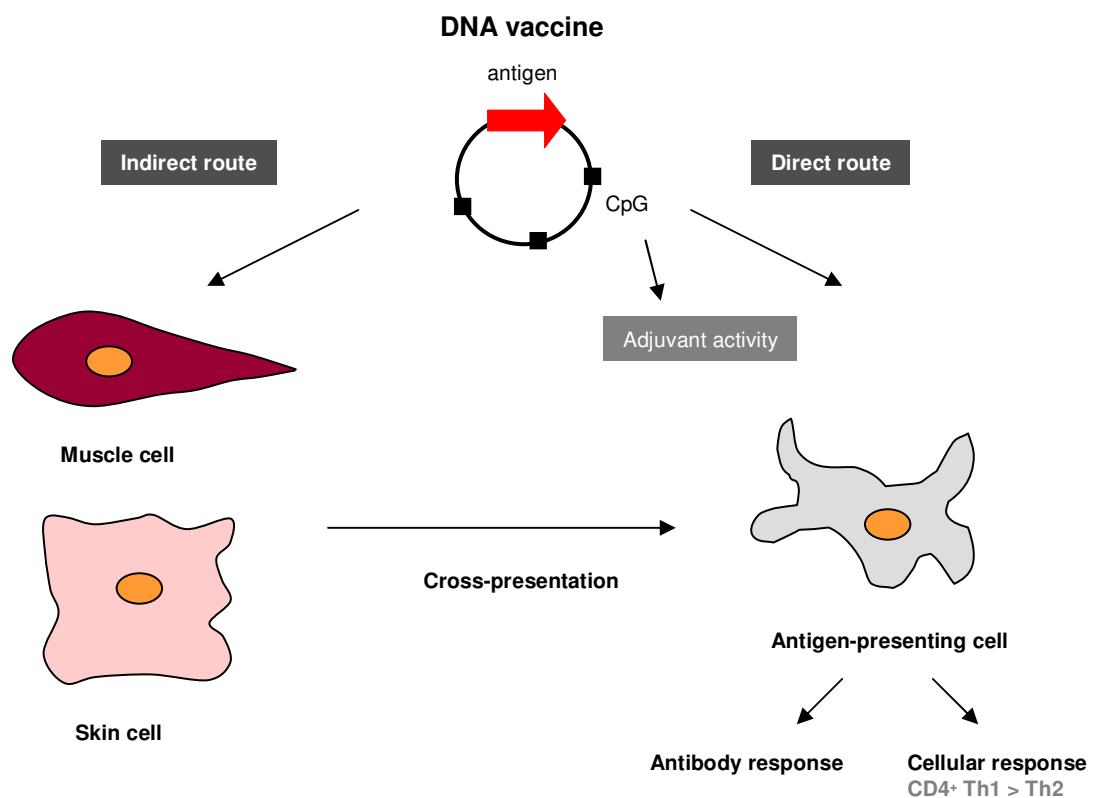


Fig. 1.2: Principle of genetic immunization. Administration of an antigen-encoding plasmid leads to transfection of muscle or skin cells or directly of APC. Transfer of the antigen to APC can also occur via cross-presentation, so that all arms of the immune response are induced. CpG sequences act as adjuvant supporting a Th1-like inflammatory response.

The flexibility of the vaccine is another advantage since elements of the plasmids can be altered easily using recombinant DNA technologies. Coexpression of cytokines or costimulatory molecules for example can enhance and modulate the immune response elicited (Iwasaki et al. 1997). Although the DNA acts as antigen depot, the immune response provoked by genetic immunization is usually weak compared to that induced by traditional vaccines (Donnelly et al. 1997). The effective transfer of the genetic material into immune cells represents the limiting factor of this method, indicating the need for new delivery systems.

1.1.3.2 Live viral and bacterial carriers

As alternative to plasmid DNA, attenuated or nonpathogenic bacteria (see section 1.2.1) and viruses can be engineered to carry foreign antigens. Gene-based vaccines consisting of recombinant viral vectors derived mainly from adenoviruses, poxviruses (e.g. vaccinia and the highly attenuated strain Ankara - MVA) and fowlpox are being investigated as vectors for DNA coding for heterologous antigens (Ada 2005). Techniques to incorporate foreign DNA into vaccinia virus were first described in 1982 (Mackett et al. 1982). Vaccinia viruses have a wide host range and can accommodate large amounts of DNA rendering them attractive for the development as multivalent vaccines. Viral vectors deliver DNA with more efficiency. Thereby they induce stronger immune responses than DNA vaccines alone (McConkey et al. 2003). However, drawbacks concerning preexisting and vector-induced immunity that limit the effectiveness of the viral vaccine must be overcome for a broad application of this type of vaccine. In addition, viral vector vaccines are demanding in terms of manufacturing (Ulmer et al. 2006).

1.1.3.3 Routes of immunization

Needles and syringes are the most commonly used means for administration of vaccines and therapeutics (Mitragotri 2005). Despite their common use, needle-based immunizations have several limitations. They are stressful, laborious, dangerous when used improperly and above all cost-intensive. Moreover, most infections, including gastrointestinal, respiratory and sexually transmitted infections enter via mucosal surfaces. To prevent these infections, the induction of a mucosal immune response is required (Holmgren and Czerkinsky 2005). Parenterally administered vaccines are inefficient to induce a protective immune response against mucosal pathogens due to the

compartmentalization of the systemic and mucosal immune system (McGhee et al. 1992). Therefore it is important to develop vaccines that induce both systemic and mucosal immune responses and can be applied mucosally by the oral, nasal, rectal or vaginal route. The oral immunization route is particularly appealing, as it offers ease of administration, high patient compliance and a long history of use for live attenuated pathogens (Mitrugotri 2005). Oral delivery of vaccines was started almost 50 years ago with the oral polio vaccination. Several other needle-free vaccines (e.g. against *Salmonella typhi*, *Vibrio cholerae* and rotavirus) are now available (Neutra and Kozlowski 2006). However, the oral administration of most vaccines has proved to be challenging, especially for non-living vaccines (that is killed pathogens, subunit, protein and DNA vaccines). Problems like the inactivation of vaccines in the gastrointestinal tract and the requirement of high doses have to be faced. A multitude of vehicles are therefore explored for oral delivery of vaccine antigens, including transgenic plants (Kong et al. 2001), viral vectors (Sullivan et al. 2000) commensal or attenuated pathogenic bacterial strains (Green and Baker 2002) as well as various inert systems. Best known among the inert systems are lipid-based structures with entrapped antigens, such as liposomes and immunostimulating complexes (ISCOMS), and mucosa-binding proteins (e.g. the binding subunits of cholera toxin or *E. coli* enterotoxin) to which antigens have been linked either chemically or as gene fusion proteins (reviewed by Holmgren and Czerkinsky 2005).

Another effort to replace administration by needles is direct application of the antigen with a strong adjuvant like cholera toxin to skin using a patch. This so-called transcutaneous immunization technique elicits rapid up-take of antigen by dendritic cells (Langerhans cells) in the epidermis and later presentation in the draining lymph nodes (Glenn et al. 2000).

1.1.3.4 Sequential immunization

The idea of “boosting” immune response is as old as the use of vaccines. Repeated administrations with the same vaccine (homologous boosting) have been shown to be effective for humoral responses. This method is sometimes inefficient in case of cellular immunity since prior immunity to the vector impairs the generation of an immune response (Woodland 2004). In contrast to the approach of giving several doses of the same preparation of an antigen to enhance immune responses, the concept arose of priming with one formulation of an antigen and boosting with a different formulation (Ada 2005). It was

shown that mice immunized with a DNA preparation and later boosted with fowlpox virus both expressing the same antigen (influenza hemagglutinin) exhibited 50-fold higher anti-hemagglutinin titers than those immunized with two injections of the same preparation (Ada 2001). The validity of this heterologous “prime-boost” approach has been confirmed and extended. Many strategies use DNA vaccines to prime and recombinant viral vectors as a booster. For example, priming with DNA and boosting with MVA induces cellular immunity against the malaria parasite *Plasmodium falciparum* (Schneider et al. 1998) and HIV (Amara et al. 2001). Heterologous prime-boost vaccination schemes starting with a bacille Calmette-Guerin (BCG) prime followed by a subunit boost consisting of protein, DNA or recombinant virus are also promising in tuberculosis vaccination schemes (Kaufmann and McMichael 2005).

1.1.4 Immunotherapy

Vaccines are, by definition, prophylactic. Besides preventing infectious diseases, the development of therapeutic treatments against cancer, chronic infections and autoimmune diseases is of utmost importance. Immunotherapy aims at mobilizing or manipulating a patient’s immune system to treat or cure disease (Steinman and Mellman 2004). Strategies range from mobilizing vaccines (so-called “active” immunotherapy) to administration of biological reagents such as monoclonal antibodies, cytokines or previously activated immune cells (“passive” immunotherapy).

1.1.4.1 Therapeutic vaccines for chronic infections

Some infectious microorganisms cause persistent infections due to evasion or subversion of immune control (Ada 2005). The treatment of such chronic infections including HIV, human papillomavirus (HPV) and hepatitis B and C virus (HBV/HCV) often fails due to ineffective antibiotic or antiviral therapy (Autran et al. 2004). Consequently, severe complications can occur – including immune deficiency caused by HIV, liver cirrhosis and hepatocarcinoma induced by HBV/HCV and cancer induced by HPV. Therapeutic vaccination is an alternative medical intervention that aims to prevent such severe complications by reinforcing host defenses. Vaccination is proposed to be most suitable after decreasing the pathogenic burden with antimicrobials (Zajac et al. 1998). Several different strategies are explored for the treatment of chronic infections including recombinant viral vectors, antigen-loaded dendritic cells (DC) (Wack and Rappuoli 2005)

as well as chemokines and cytokines used as adjuvants to enhance T cell function and maintenance (Autran et al. 2004).

1.1.4.2 Therapeutic vaccines for cancer therapy

There are two types of cancer from the vaccine standpoint. One type is associated with viral infections, such as genital carcinomas that are associated with HPV infections. Consequently, it was demonstrated that vaccination against the virus prevents tumorigenesis. The development of the recently licensed prophylactic HPV vaccine is the outstanding success story (Schiller and Lowy 2006). Other types of cancers amenable to vaccines include spontaneous tumors such as melanoma that express endogenous tumor antigens. Immune cells play an important role in the control of such malignancy (Shankaran et al. 2001). Innate immune cells respond to “danger” signals, which can be provided by growing tumors due to cell transformation and disruption of the surrounding microenvironment. Ideally, these signals induce inflammation, activation of innate effector cells with antitumor activity and stimulation of DC to present tumor-derived antigens and to trigger an adaptive immune response (reviewed by Blattman and Greenberg 2004). Tumors often exhibit strategies to escape this immunosurveillance, such as exclusion of immune cells from tumor sites, impairing antigen presentation by DC and poor immunogenicity due to reduced expression of MHC molecules and costimulatory proteins (Marincola et al. 2000). Even if a response is induced, tumor cells may escape elimination due to immunoediting, e.g. by losing targeted antigens or rendering reactive T cells anergic. In addition, breaking self-tolerance is an important challenge in developing cancer vaccines because the targets may be self-molecules as opposed to foreign antigens (Henderson et al. 2005).

Therapeutic vaccines against cancer that manipulate T cell immunity can consist of tumor cells, lysates thereof or defined antigens. Antigens can be delivered as proteins, liposomes, by viral and bacterial vectors or *ex vivo* treated DCs (Yannelli and Wroblewski 2004). Since the preparation of antigen-loaded DCs from individuals with cancer is a laborious and costly procedure, it is desirable to develop vaccines, which deliver antigens and activation signals directly to DCs (Collins and Cerundolo 2004). An alternative to these immunogenic vaccines has been the isolation of autologous tumor-reactive T cells, expansion of cells *in vitro* and return into the patient. It is also possible to genetically modify T cells before infusion to increase tumor-specificity, tumor-homing and effector

functions (Kershaw et al. 2005). Moreover, strategies to manipulate the innate immune response by administration of adjuvants, cytokines or ligands for costimulatory proteins directly triggering innate immune cells have been developed (Blattman and Greenberg 2004). As the global activation of the innate immune system often leads to toxicity, it is desirable to combine this approach with specific targeting of the tumor, e.g. provide effector molecules like TNF α specifically at the tumor site. Passive immunotherapeutic approaches have been explored in terms of humoral immunity, for instance the administration of monoclonal antibodies directed against cell-surface proteins on tumor cells (von Mehren et al. 2003). These antibodies can bind to tumors and activate effector cells, e.g. by mediating antibody-dependent cell-mediated cytotoxicity (ADCC), or interfere with tumor growth by blocking survival or inducing apoptotic signals. For example, a monoclonal antibody approved for clinical use reacts with the Her-2/Neu receptor on breast cancer cells thereby blocking growth signals (Rueckert et al. 2005).

Despite these promising results in developing immunotherapeutic treatments for cancer a strong need for practical immunization strategies remains. In this respect, the use of bacteria as vaccine vectors and delivery systems for therapeutic molecules represents a very promising alternative.

1.2 Bacteria as delivery systems for prophylactic and therapeutic molecules

1.2.1 Bacteria as antigen delivery systems

Antigen delivery systems often suffer from low efficiencies, safety concerns and/or demanding manufacturing processes. Moreover, it is desirable to develop carriers for mucosal application of vaccines to trigger appropriate immune responses against the vast amount of pathogens that enter the host via mucosal surfaces. In this respect, live bacterial vectors represent an alternative to the commonly used systems due to several advantages. First of all, they offer simple and inexpensive handling and production as well as the possibility to administer them as lyophilized organisms abrogating the need for a cold chain. Bacteria are easy to manipulate so that improved strains with desired properties can be generated in a short time. In addition, bacterial components, e.g. lipopolysaccharides, lipoproteins, flagella and bacterial DNA possess strong adjuvant properties as they are recognized via TLRs (Aderem and Ulevitch 2000). Another advantage is the ability to

control bacterial live vaccines with antibiotics in case of undesired side effects after administration.

Enteropathogenic bacteria are the main group to be explored for the mucosal delivery of protein and DNA vaccines. They are able to enter the gut mucosa after oral application and target immune inductive sites of the lymphoid tissues of the gut and the periphery (Shata et al. 2000). Therefore, resembling a natural infection, oral immunization with such bacterial strains should stimulate the mucosal and systemic, humoral and cellular compartments of the immune system. Attenuation of these pathogenic strains can be accomplished through the deletion of different genes, including virulence, regulatory and metabolic genes. Thereby an appropriate balance between minimal toxicity and maximal immunogenicity has to be found in order to develop effective and safe carriers for delivering antigens to the immune system. The antigen can be encoded by a prokaryotic expression plasmid or chromosomally integrated and by this means expressed by the bacteria themselves. Alternatively, bacteria harboring eukaryotic expression plasmids can be used as carrier for genetic immunization.

1.2.1.1 Bacteria-mediated delivery of heterologous antigens

There are currently three live bacterial vaccines commercially available: *Salmonella enterica* serovar Typhi Ty21a, *Vibrio cholerae* CVD 103-HgR and *Mycobacterium bovis* BCG. These strains and many other commensal and attenuated strains have been used as vectors for the expression of foreign (heterologous) antigens derived from bacterial, fungal, viral or parasitic organisms (reviewed by Kotton and Hohmann 2004). The use of attenuated pathogens seems to be especially attractive, since the limited infection established by the attenuated strain leads to the induction of natural immune responses in the host specific for the carrier itself as well as the heterologous antigen (Medina and Guzman 2001). Live recombinant bacteria such as *Salmonella* spp. (see section 1.3.3.1), *Vibrio cholerae* (Butterton et al. 1995), *Yersinia enterocolitica* (Gundel et al. 2003), *Shigella flexneri* (Koprowski et al. 2000), *Escherichia coli* (Critchley et al. 2004) and *Listeria monocytogenes* (Gentshev et al. 2000) have been explored as potential vaccine delivery systems for foreign antigens. Although such live bacterial vectors have been shown to induce humoral and cell-mediated immune responses against passenger antigens in animal and human studies, there are at this stage no licensed vaccines in clinical use.

In particular, the bacteria that are able to replicate and release antigen within the cytosol of host cells are attractive candidates for the elicitation of cell-mediated immunity against passenger antigens. *Shigella flexneri* is a gram-negative bacterium that targets the lymphoid tissues in the colonic mucosa and infects phagocytic and epithelial cells of the gut. Within host cells, it efficiently escapes the endosomes and directly accesses the cytoplasm (Cossart and Sansonetti 2004). The gram-positive bacterium *Listeria monocytogenes* exhibits similar properties as it expresses the pore-forming toxin listeriolysin O (LLO), which is activated at low pH and reducing environment in the phagosome (Gentshev et al. 2001). The bacteria are thus able to enter the cytosol, move around due to recruitment of host cell actin and spread from cell to cell (Tilney and Portnoy 1989). *Shigella* and *Listeria* are therefore able to replicate and grow within the cytosol of the eukaryotic host cell, where they are protected from extracellular and phagosomal immune responses (Mollenkopf et al. 2001). Consequently, secreted proteins can directly enter the endogenous MHC class I antigen processing pathway leading to the stimulation of CTL.

1.2.1.2 Bacteria-mediated delivery of DNA

Recently, intracellular bacteria have also been recognized as carriers for the delivery of eukaryotic expression plasmids to mammalian cells *in vitro* and *in vivo*. The first direct transfer of plasmid DNA from bacteria to mammalian cells was described in 1980 (Schaffner 1980) when tandem copies of the SV40 virus genome carried by non-invasive *E. coli* were transferred into cocultured mammalian cells. Since then, several other, mostly invasive bacteria have been studied for gene transfer, most extensively recombinant *Salmonella enterica* spp. (see section 1.3.3.2), *Shigella flexneri* and *Listeria monocytogenes* (reviewed by Loessner and Weiss 2004). Using live bacterial carriers for genetic immunization combines the advantages and versatilities of both vaccination approaches. In addition, the amount of plasmid required for bacteria-mediated DNA transfer is small compared to injection of naked DNA, since bacteria directly target immune inductive sites of the host. Moreover, bacteria can be used to deliver therapeutic molecules (e.g. cytokines) or genes to complement genetic defects (Loessner and Weiss 2004). In contrast to delivery of foreign proteins, delivery of DNA allows also the expression of post-translationally modified antigens (Devico et al. 2002). In comparison to most viral DNA carriers, bacteria are easy to manufacture and safe in use. The existence of established attenuated bacterial strains and effective antibiotics in case of complications

represent an important advantage over viral delivery systems (Schoen et al. 2004). In addition, the tissue tropism of certain bacteria can be employed for specific targeting. Cellular spreading occurs for instance in the case of *Listeria* and *Shigella* thereby reaching tissue layers *in vivo* that are potentially inaccessible to viral vectors.

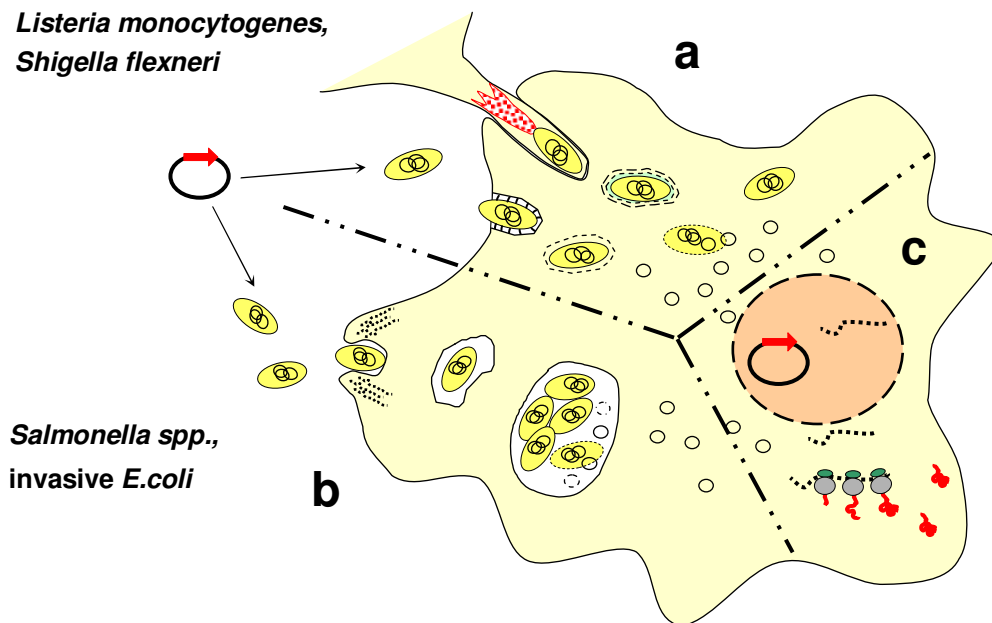


Fig. 1.3: The intracellular tropism of bacterial DNA vectors. (a) *Shigella* and *Listeria* are cytosolic replicating bacteria that are able to escape the vacuolar compartment, replicate inside the host cell cytosol and spread to neighboring cells via protrusions and invaginations. DNA transfer occurs following lysis of the bacteria in the cytosol. (b) Intracellular bacteria like *Salmonella* and invasive *E. coli* remain in the phagosome where bacterial replication ensues, DNA transfer into the cytosol occurs by an unknown mechanism. (c) In both cases, the plasmid reaches the nucleus, where the antigen is transcribed and later, in the cytosol, translated into protein.

The transfer of DNA from different bacteria to the nucleus of mammalian cells is hampered by several barriers (Fig. 1.3). After invasion of the host cell, *Listeria* and *Shigella* escape from the phagocytic vacuole to the cytoplasm, whereas invasive *E. coli* and *Salmonella* are localized in phagosomes (Schoen et al. 2004). In both cases, the release of the eukaryotic expression plasmid into the cytosol or the phagosome is a prerequisite for gene transfer. In order to achieve this, bacterial cell death and lysis has to occur. This can be induced due to the attenuation of the carrier, bacteriophage lysis components or

antibiotics (Loessner and Weiss 2004). Upon translocation into the nucleus of the host cell, the plasmid-encoded antigen is expressed and can induce humoral and cellular immune responses against bacterial, viral and tumor antigens (reviewed by Vassaux et al. 2006).

The first functional gene transfer regarding subsequent production of a functional foreign protein was observed using an attenuated *Shigella flexneri* strain (Sizemore et al. 1995). To attenuate the bacterial vector, a deletion mutation in the *asd* gene encoding aspartate β -semialdehyde dehydrogenase was introduced (Noriega et al. 1994). This essential enzyme is required to synthesize the bacterial cell wall constituent diaminopimelic acid (DAP). A lack of DAP leads to lysis of growing bacteria and release of macromolecules (Loessner et al. 2006). Using this modified strain, liberation of a β -galactosidase reporter plasmid in human cells as well as subsequent expression of the plasmid encoded *lacZ* gene could be demonstrated *in vitro* (Sizemore et al. 1995). Various other metabolically attenuated strains of *S. flexneri* have been demonstrated to successfully deliver plasmid DNA *in vitro* in the meantime (Sizemore et al. 1997, Loessner and Weiss 2004). Since these bacteria are not invasive in the mouse intestine, intranasal application was used to investigate its potential as carrier for DNA vaccines *in vivo*. By this route, *Shigella* mediated delivery of DNA vaccines has been shown to induce cellular and humoral immune responses against a range of antigens (Sizemore et al. 1997, Fennelly et al. 1999, Vecino et al. 2004).

Similarly, an attenuated *L. monocytogenes* strain has been engineered to undergo self-destruction by production of a phage lysine under the control of the listerial promoter P_{actA} , which is preferentially activated in the cytosol (Dietrich et al. 1998). This bacterial vector could deliver functional plasmids in mouse macrophage cell lines. In addition to these first experiments, *in vitro* gene transfer into a broad range of cells has been described for *L. monocytogenes*, including cell lines of epithelial origin, murine primary macrophages as well as human DCs (Hense et al. 2001, Krusch et al. 2002, Pilgrim et al. 2003). However, high bacterial doses were required to achieve DNA transfer. A recent study showed that such low rates of *Listeria*-mediated transfection are not due to poor invasion frequency, bacterial lysis or DNA degradation but to association of the plasmid DNA with macromolecules or organelles, which limits gene transfer to the nucleus and consequently its expression (Zelmer et al. 2005).

Alternatively, *E. coli* that are usually not invasive were manipulated to effectively deliver DNA to mammalian cells. Transformation of DAP-auxotroph *E. coli* with the virulence plasmid of *S. flexneri* rendered the bacteria able to enter epithelial cells and deliver plasmid DNA to their host cells (Courvalin et al. 1995). Grillot-Courvalin et al. further demonstrated that invasive *E. coli* carrying the invasion gene *inv* from *Yersinia pseudotuberculosis* were able to transfer DNA more efficiently than non-invasive bacteria (Grillot-Courvalin et al. 1998). Co-production of LLO together with the invasion gene by recombinant *E. coli* further increased the DNA transfer *in vitro* in several epithelial cell lines, indicating that bacterial escape from the phagosome might improve gene transfer efficiency. More recently, transfer of DNA using recombinant *E. coli* was also demonstrated *in vivo* (Castagliuolo et al. 2005).

1.2.2 Bacteria in cancer treatment

New effective and better tolerated cancer treatments are urgently needed. They should replace current relatively toxic regimes of chemo- and radiotherapy (Theys et al. 2003). In this respect, bacteria that are genetically engineered to express a specific therapeutic gene specifically in the tumor microenvironment represent a powerful treatment modality. The preferential accumulation of bacteria in certain experimental tumors was initially reported in the 1950s when spores of *Clostridium tetani* were shown to germinate exclusively in the tumor after intravenous administration into tumor-bearing mice (Malmgren and Flanigan 1955). It was assumed that the obligate anaerobic bacteria were replicating in the necrotic/hypoxic centers of these tumors, leaving the well-oxygenated normal tissues unaffected. Thereafter, studies involving different strains of Clostridia led to the conclusion that bacterial growth was associated with “oncolysis” of large tumors with a necrotic/hypoxic centre but had little effect on metastatic lesions. Moreover, some animals became ill and died during the peak of oncolysis probably due to bacterial inflammation and the release of necrotic tumor debris (reviewed by Theys et al. 2003). In an initial clinical trial in humans using spores of Clostridia incapable of producing toxins, most patients showed no objective regression (Carey R.W. et al. 1967). Thus, further studies were abandoned due to the lack of clinical efficiency. Nevertheless, these initial studies have supported the idea to use bacteria for the selective delivery of anticancer agents to tumors.

More recently, investigators have attempted to use the tumor-targeting properties of Clostridia for the selective delivery of pro-drug converting enzymes (Minton 2003). For instance, the *E. coli* enzyme cytosine desaminase was expressed and shown to convert the non-toxic pro-drug 5-fluorocytosine (5-FC) into the toxic chemotherapeutic compound 5-fluorouracil (5-FU). This product diffuses in the tumors and kills cancer cells through a bystander effect. Using a similar principle, a Clostridium strain was engineered in which a radio-responsive promoter drove the expression of TNF- α (Nuyts et al. 2001a).

An optimized strain devoid of its lethal toxin (*C. novyi*-NT) could eradicate large established tumors in mice in the absence of additional chemotherapy and radiation due to the induction of a potent immune response (Agrawal et al. 2004). Once germinated within the tumor, these Clostridia destroy adjacent tumor cells through the secretion of degradative enzymes, at the same time the host reacts to the bacterial infection by producing cytokines that lead to the influx of inflammatory cells. The inflammatory reaction restricts on the one hand bacterial growth; on the other hand it may also contribute to the destruction of tumor cells. Moreover, it might stimulate a cellular immune response, which could destroy tumor cells not lysed by the bacteria. Whether a tumor is cured depends on the balance between bacteriolysis, angiogenesis, regrowth of residual tumor cells and the rate of development of the immune response (Agrawal et al. 2004). The same attenuated Clostridia strain and its membrane-disrupting properties was recently used to enhance the release of the liposome-encapsulated toxic drug doxorubicin within a tumor thereby mediating complete eradication of the established tumor (Cheong et al. 2006).

Anaerobic Bifidobacteria have also been investigated and shown to colonize tumors. In contrast to Clostridia, Bifidobacteria are non-pathogenic bacteria found naturally in the digestive tract of humans and other mammals and therefore may represent a safer alternative compared to Clostridia (reviewed by Vassaux et al. 2006). First studies with Bifidobacteria described colonization of several types of mouse tumors after systemic administration. However, no antitumor effects could be observed (Kimura et al. 1980). More recently, strains of these bacteria were modified to produce cytosine desaminase and the antiangiogenic protein endostatin (Michl and Gress 2004). The latter approach resulted in inhibition of angiogenesis and retardation of growth of the tumor after systemic administration. In addition, oral administration of *B. longum* carrying the endostatin gene was efficient in a liver tumor model (Fu et al. 2005).

In contrast to anaerobic Clostridia and Bifidobacteria, facultative anaerobic bacteria have also been tested in tumors. They have the potential to colonize not only the anaerobic necrotic parts of the tumor but also oxygenated proliferative and quiescent tumor regions as well as metastatic lesions (Forbes et al. 2003, Saltzman 2005). Selective accumulation in tumors of immunocompromised and immunocompetent rodents has been observed after intravenous administration of *Salmonella enterica* serovar Typhimurium (*S. typhimurium*), the laboratory strain *E. coli* DH5 α and attenuated *Vibrio cholerae* (Yu et al. 2004). Such bacteria were engineered to encode light-emitting proteins, which might be applied as diagnostic tool for the detection of tumors and metastases in the future. Invasive *E. coli* were used to deliver functional, natural *E. coli* proteins to mouse tumors (Critchley et al. 2004). Thereby, a therapeutic effect of the product of the *E. coli* gene *deoD* was observed in association with the prodrug 6-MPDR (6-methylpurine-2'-deoxyriboside). However, the bacteria were injected directly into the tumor in this study. Thus, *E. coli* awaits to be explored in detail as vector for targeted delivery of therapeutic molecules to tumors. In contrast, extensive research is carried out using attenuated strains of *S. typhimurium* for tumor therapy, as discussed below.

1.3 **Salmonella as vectors for vaccination and tumor therapy**

Salmonella are gram-negative, facultative anaerobic, rod-shaped bacteria, which belong to the group Enterobacteriaceae and are established as one of the most important causes of food borne illness worldwide (Adams and Moss 2000). They are transmitted via the fecal-oral route and depending on the species variant cause disease in animals or man. In humans, gastroenteritis, correlated with serovar Typhimurium and Enteritidis, and a systemic infection, provoked by serovar Typhi and Paratyphi, can be distinguished. Whereas gastroenteritis proceeds mostly harmless, serovar Typhi and Paratyphi belong to life-threatening infectious agents (Ohl and Miller 2001). *S. typhimurium* is the causative agent of murine typhoid fever and serves as experimental model for human typhoid fever (Hapfelmeier and Hardt 2005). This mouse model has been of great importance for the investigation of virulence mechanisms and of immune responses against *S. typhimurium*.

1.3.1 **The course of a Salmonella infection**

After oral uptake, Salmonella pass the acidic environment of the stomach and reach the gut (Rychlik and Barrow 2005). Invasion of the gut mucosa occurs mainly via M cells of the

follicle-associated epithelium (FAE) in the Peyer's Patches (PP) of the ileal portion of the small intestine as ports of entry (Jones et al. 1994). M cells are specialized for the transepithelial up-take of macromolecules, particles and microorganisms, such that antigens can cross the epithelial barrier of the gut and induce mucosal immune responses (Neutra et al. 1996). Salmonella selectively adhere to M cells and initiate signal transduction pathways that induce their uptake and subsequent translocation to the basolateral side of the epithelial cell (Fig. 1.4).

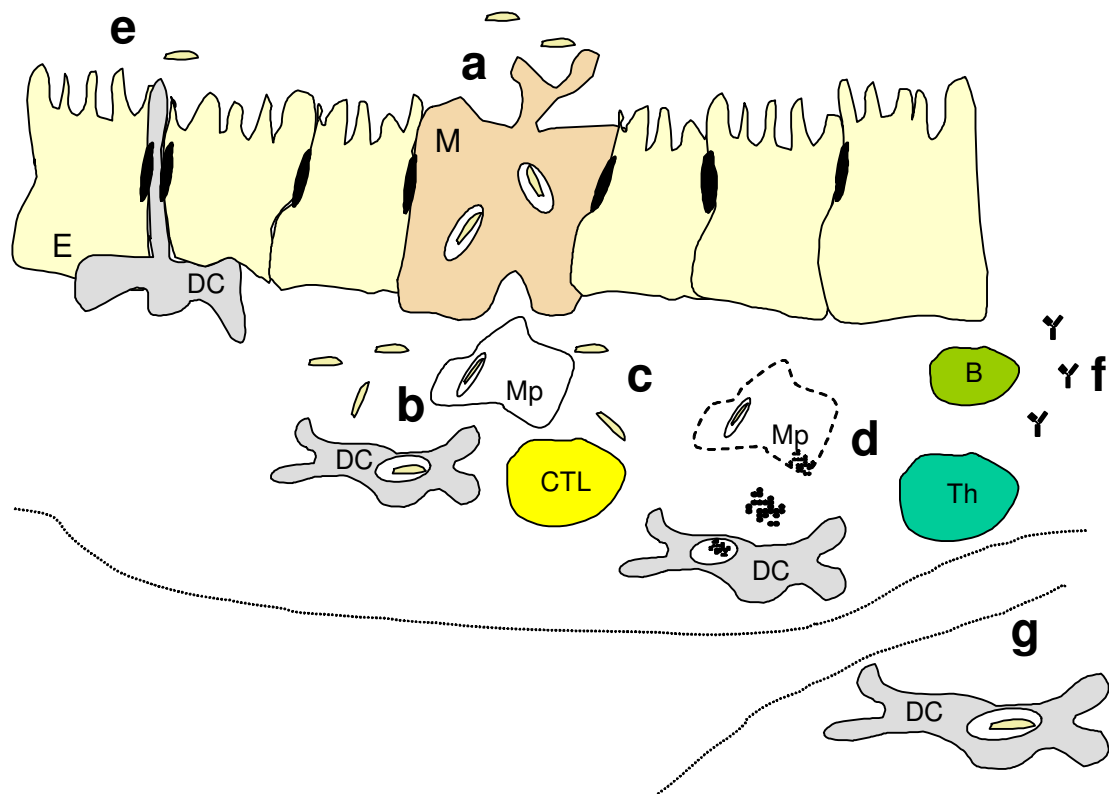


Fig. 1.4: Course of a Salmonella infection. (a) Salmonella are taken up by M cells (M) of the PP and (b) invade DC and macrophages (Mp) in the subepithelial dome (SED). (c) Dead macrophages are phagocytosed by DC. (d) Infected DC stimulate T cells (CTL, Th). (e) Direct uptake of Salmonella by intraepithelial DC. (f) Secretion of Salmonella-specific antibodies by B cells (B). (g) Migration of infected phagocytes to other organs.

This event is triggered by genes located on Salmonella pathogenicity island 1 (SPI-1), a distinct region of the Salmonella chromosome (Cossart and Sansonetti 2004). SPI-1 encodes a type III secretion system (TTSS) that is expressed due to high osmolarity and low oxygen tension when the bacteria are moving extracellularly in the gut lumen before the first encounter with host cells. Therefore, the bacteria target the M cells as “loaded

weapons” equipped with the syringe-like TTSS apparatuses as well as a pre-formed pool of effectors (Schlumberger and Hardt 2006). Upon encounter of M cells, the effector proteins are delivered into the host cell cytoplasm by the translocon complex consisting of proteins SipB and SipC. The injected effectors reorganize the cytoskeleton of the cell to cause localized membrane ruffling, bacterial engulfment into membrane-bound vesicles and transcytosis (Ohl and Miller 2001). After transcytosis, the bacteria reach the dome area of the organized mucosa associated lymphoid tissue (MALT), which is the inductive site of the mucosal immune system (Brandtzaeg and Pabst 2004). Here, bacteria are taken up by phagocytic cells, mainly resident and recruited macrophages and DC (Richter-Dahlfors et al. 1997). Alternatively to this process, *Salmonella* can also directly enter intestinal cells by the apical pole of the cell (reviewed by Abrahams and Hensel 2006) or be captured by DC that emit pseudopods between epithelial cells (Rescigno et al. 2001).

During evolution, *Salmonella* have developed several strategies to resist innate immune defenses to survive phagocytosis and establish infection. Initially, *Salmonella* can cause macrophage apoptosis through activation of caspase-1, thereby also triggering the production of pro-inflammatory cytokines like IL-1 β and IL-18 (Hueffer and Galan 2004). The elicited inflammatory reaction leads to the recruitment of polymorphonuclear cells (PMN) and simultaneously disintegrates the epithelial lining to allow their transmigration to the gut lumen. At the same time inflammation helps bacteria to cross the epithelial barrier (Sansonetti 2004). Additionally, *Salmonella* express a second TTSS encoded in SPI-2, which is of key importance for their ability to replicate and survive inside host cells (Schlumberger and Hardt 2006). Different regulatory systems are responsible for the activation of the second TTSS within the vacuolar environment where *Salmonella* resides (Bijlsma and Groisman 2005). First, the SPI-2 encoded local regulatory system SsrA/B, which is activated by low Ca²⁺ levels, acidic pH and low osmolarity, induces genes encoding the components of the SPI-2 TTSS and SPI-2 effectors (Garmendia et al. 2003). Furthermore, the global regulatory system PhoP/Q has been identified to modulate the expression of SPI-2 genes by regulating expression of the regulator SsrB (Bijlsma and Groisman 2005). The transcriptional regulator PhoP itself is activated by PhoQ mediated phosphorylation that occurs when the sensor protein PhoQ encounters low Mg²⁺ levels in the vacuolar environment (Lejona et al. 2003).

The second TTSS allows injection of effector proteins from the phagosome into the host cell cytoplasm, thereby enabling bacteria to modify the vacuole into a *Salmonella*-containing vacuole (SCV). A key role plays the SPI-2 effector SifA, which is required for the recruitment of vesicles to increase the amount of SCV membrane in order to avoid rupture of the vacuole and transition into a lysosome (Cossart and Sansonetti 2004). This strategy allows further dissemination via infected phagocytes to deep organs, such as lymph nodes, liver and spleen.

The cells of the innate immune system play an essential role in the early responses to *Salmonella*, especially macrophages and polymorphonuclear neutrophils (Vassiloyanakopoulos et al. 1998). Stimulation of these cells by ligands of TLR (e.g. LPS, lipoprotein, flagellin) and *Salmonella* effectors gives rise to pro-inflammatory cytokines including IFN- γ and TNF- α (Galan 2001). IFN- γ and TNF- α lead to activation of anti-microbial effector mechanisms and impair bacterial replication within macrophages (Gulig et al. 1997). Although the innate immune system can initially control *Salmonella* infection, the acquired immune response is required for clearance of the infection as well as for protection against reinfection (Mittrucker and Kaufmann 2000). T cells are of special importance, particularly Th1 CD4⁺ cells, whose induction is stimulated by the pro-inflammatory cytokine environment (Pie et al. 1997). In addition to T cell-mediated immunity, the production of antibodies has been proposed to be important in mediating immunity to *Salmonella* (Mittrucker et al. 2000). The induction of *Salmonella*-specific CD4⁺ T cells and CD8⁺ T cells is dependent on the capacity of DC to process *Salmonella* antigens for presentation on MHC class I and MHC class II (Svensson et al. 1997, Svensson et al. 2000, Yrlid and Wick 2002). Thus, *Salmonella*-infected DC in the subepithelial dome (SED) of the PP migrate to the interfollicular region to present the antigen to T cells (Ravindran and McSorley 2005). Moreover, *Salmonella*-induced apoptosis of macrophages provides a reservoir of antigens that can be presented by bystander DC on MHC-I and MHC-II (Yrlid and Wick 2000), a process called cross-presentation. In addition, *Salmonella* entry into the PP may lead to the release of soluble bacterial antigens into the SED, which can be acquired by DC and presented or alternatively result in activation of *Salmonella*-specific IgA secreting B cells (Ravindran and McSorley 2005). Migrating DC may also lead to the generation of systemic responses. Thus, during infection, a bacteria specific effector population expands rapidly in lymphoid

tissues and redistributes effectively to the major sites of bacterial infection mediating bacterial clearance (Mittrucker and Kaufmann 2000).

1.3.2 Attenuation of *Salmonella* for the use as live vector

Previously, inactivated whole-cell vaccines have been used to provide protection against typhoid fever in man. Due to the high incidence of adverse reactions, these vaccines are considered to be unsuitable for use as public health vaccines. The licensed live attenuated oral vaccine strain *S. enterica* serovar Typhi strain Ty21a has provided an alternative (Germanier and Fuer 1975). This strain was generated by chemical mutagenesis and has been evaluated in several efficacy trials and shown to be safe and effective, though it requires several doses to elicit protection (Levine et al. 1987, Ferreccio et al. 1989, Black et al. 1990).

The mouse typhoid model was extensively used in the search for attenuated strains with defined mutations (Pasetti et al. 2003). Recent progress in the understanding of the genetics of *Salmonella* virulence as well as recombinant DNA technology helped identifying target genes involved in house-keeping, biosynthesis of structural components and metabolites, pathogenesis, and bacterial resistance to host-defense mechanisms (Garmory et al. 2002). Target genes for the development of *S. typhimurium* vaccine strains include heat-shock protein *htrA* (Chatfield et al. 1992b), global regulatory components *cya/crp* and *phoP/phoQ* (Curtiss, III and Kelly 1987, Galan and Curtiss, III 1989, Mastroeni et al. 2001), amino acid biosynthesis pathway *aro* (O'Callaghan et al. 1988) and the transcriptional regulator *rfaH* (Nagy et al. 2006). Such attenuated strains invade the gut mucosa and reach the MALT as well as lymph nodes and spleen thereby inducing a protective immune response protecting against a challenge infection. But they do not cause adverse clinical responses or bacteremia.

Mutations in the bacterial prechorismate pathway belong to the best characterized attenuations for *Salmonella* vaccine strains. Prechorismate or *aro* mutants do not produce chorismate, an essential intermediate in the *de novo* synthesis of aromatic compounds including aromatic amino acids (Dunstan et al. 1998). *Salmonella typhimurium* strain SL7207 Δ *aroA* carries a non-reverting mutation in the gene *aroA* encoding for the enzyme 5-enolpyruvylshikimate-3-phosphate synthase, which results in a block of the aromatic biosynthesis pathway (Hoiseth and Stocker 1981). This mutation renders the bacteria

auxotrophic for the metabolites para-aminobenzoic acid and 2, 3-dihydroxybenzoate that are synthesized from chorismate. Since these compounds are not available in vertebrate tissues, such auxotrophic mutants cause only a mild infection, after which the bacteria are cleared by the immune system (Hormaeche 1991). Nevertheless, these derivatives confer protection against challenge with a virulent strain (Hoiseth and Stocker 1981) showing their suitability as live vaccine. In addition, the safety of such live vaccines can be increased by introducing a mutation in a second locus thereby reducing the odds of reversion (Karem et al. 1995). As established live vaccines, *Salmonella* are promising candidates for the delivery of foreign proteins and DNA for vaccination and therapeutic applications.

1.3.3 *Salmonella* as antigen delivery system

Orally administered live attenuated *Salmonella* ssp. are promising delivery systems since they are safe and highly immunogenic and can elicit long-lasting protective systemic and mucosal immune responses against heterologous antigens (Bumann et al. 2000). Moreover, they have been extensively studied regarding the delivery of DNA (reviewed by Loessner and Weiss 2004). For both applications it is advantageous that *Salmonella* can be genetically manipulated by simple means. When applied orally, such recombinant attenuated vaccine strains harboring either eukaryotic or prokaryotic expression plasmids invade the gut mucosa and eventually reach the MALT. Here, they target professional APC, therefore delivering the cargo directly to immune inductive sites (Fig. 1.5).

Bacterial components lead to activation of the phagocytic cells, which involves production of cytokines, expression of co-stimulatory molecules and surface expression of MHC molecules (Blander and Medzhitov 2006). At the same time, the bacteria reside in the phagosomal compartment until they die due to their metabolic attenuation or host cell defense mechanisms (Darji et al. 2000). In case of protein delivery, the passenger antigens are processed for presentation on MHC class I and MHC class II by activated DC. In case of DNA delivery, the expression plasmid is liberated after bacterial lysis and transferred to the nucleus (Loessner and Weiss 2004). Expression of the antigen leads to presentation on MHC class I and the stimulation of CTL by activated DC. In addition, DC can present antigen via cross-presentation after phagocytosis of apoptotic infected macrophages, thus efficiently inducing also helper T cells (Darji et al. 1997). Free antigen from dying macrophages might also be responsible for the induction of humoral responses. Infected or

antigen-carrying DC, migrating from PP to lymph nodes and spleen via the bloodstream and lymphatics, most likely cause systemic immune responses.

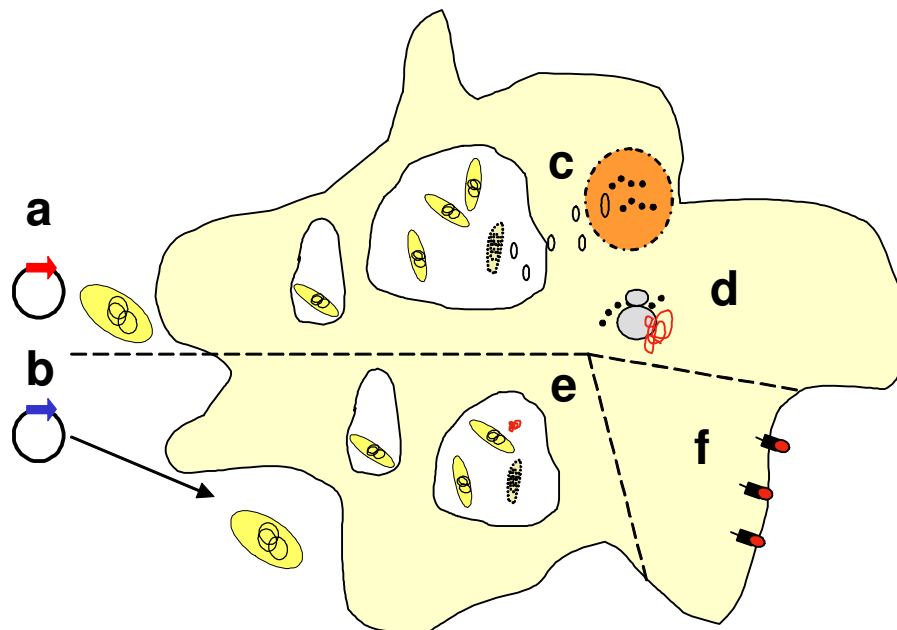


Fig. 1.5: Principles of Salmonella-mediated delivery of DNA or proteins. Attenuated *Salmonella* are transformed with a eukaryotic (a) or prokaryotic (b) expression plasmid for the delivery of DNA or protein, respectively. After infection of the APC, *Salmonella* die within the phagosome due to the attenuation leading to liberation and transfer of the DNA vaccine to the nucleus (c) and subsequent expression and presentation of the antigen (d, f). In case of protein delivery, the passenger antigen is expressed by the bacteria (e) and after bacterial lysis presented (f).

1.3.3.1 *Salmonella*-mediated delivery of heterologous antigens

Salmonella were among the first bacteria used as recombinant vectors for antigen delivery (Curtiss, III 2002) and up to now numerous studies have been performed in mice and man. In mice, there is an increasing body of evidence suggesting that live attenuated *S. typhimurium* constitute an effective delivery system for recombinant antigens from a wide variety of pathogens (Garmory et al. 2003) including parasitic, bacterial and viral antigens (Ben Yedidia et al. 1999, Igwe et al. 2002, Baud et al. 2004). The recombinant *Salmonella* have been shown to elicit humoral, secretory and cell-mediated immunity, thereby conceivably providing the capability to elicit the immune responses necessary for the protection against the heterologous antigen (Mollenkopf et al. 2001). Most of these studies used oral, intraperitoneal or intravenous immunizations, but alternative mucosal

routes such as intranasal, vaginal and rectal have also been employed successfully (Pasetti et al. 2003). However, human clinical trials using *S. typhi* showed mixed results so far (reviewed by Garmory et al. 2002). Some vaccines were able to induce detectable immune responses against heterologous antigens while others have not. In a human study with *phoP/phoQ*-deleted *S. typhimurium* expressing *H. pylori* urease, oral immunization induced urease-specific immune responses in 50% of the volunteers (Angelakopoulos and Hohmann 2000) in contrast to a previous trial with *phoP/phoQ*-deleted *S. typhi* expressing the same antigen (DiPetrillo et al. 1999). This could probably be attributed to a greater stability of the antigen-expressing plasmid in the *S. typhimurium* carrier and/or prolonged intestinal colonization.

Stability of the recombinant phenotype is one of the major limitations that have been encountered during the development of Salmonella for the delivery of foreign antigens. An efficient vaccination depends on the ability of the vector to present the foreign antigen to the immune system in a stable manner and sufficient amount, since immune responses are generally dose-dependent (Zinkernagel et al. 1997). On the other hand, vigorous antigen expression represents an additional metabolic burden for the bacteria, especially when plasmid-based systems are used, resulting in plasmid loss, reduced viability, invasiveness and immunogenicity (Medina and Guzman 2001). Other problems besides plasmid stability include low immunogenicity of the antigen due to its location and vector immunity against Salmonella, whereby the latter is controversial. Reports suggested that prior experience with the vaccine strain might be able to potentiate subsequent immune responses (Bao and Clements 1991, Whittle and Verma 1997), whereas other studies showed impaired immune responses in situations of pre-existing immunity (Attridge et al. 1997, Attridge and Vindurampulle 2005). Thus, this issue requires further studies in order to understand the impact of vector immunity for the further use of Salmonella as carrier for heterologous antigens but also plasmid DNA.

1.3.3.2 Salmonella-mediated delivery of plasmid DNA

Salmonella remains in the endosomal compartment of infected cells. Therefore it is puzzling how these bacteria transfer eukaryotic expression plasmids to host cells and consequently mediate a high level of reporter gene activity (Darji et al. 1997, Paglia et al. 1998). Oral administration of a single dose of the auxotrophic mutant strain SL7207 bearing the plasmid-encoded *lacZ* gene under control of the cytomegalovirus (CMV)

immediate-early promoter elicited high levels of β -galactosidase specific antibodies, T cell proliferation and CTL response in BALB/c mice (Darji et al. 1997). In comparison, the Salmonella DNA vaccine induced superior immune responses than recombinant bacteria expressing the same antigen constitutively at high levels. In the same study, protection of mice against a lethal challenge with *L. monocytogenes* was observed using the virulence factor LLO as antigen. These findings could be confirmed in outbred strains and mouse strains more resistant to Salmonella than BALB/c mice, although several administrations of the bacteria were needed to induce CTL, T-helper cell and antibody responses (Darji et al. 2000). In this study, Salmonella-mediated genetic immunization induced strong systemic humoral and cell-mediated immune responses after oral application, but hardly any antigen specific secretory IgA antibodies could be detected. Thus, the cells that are transfected by Salmonella in the PP might not initiate antibody production and/or the switch of B cells to IgA (Urashima et al. 2000). Viral infections have also been targeted through DNA vaccination by *S. typhimurium*. Oral immunization with a strain encoding hepatitis B virus surface antigen has been shown to elicit CTL in BALB/c mice (Woo et al. 2001). Moreover, vaccination against HIV was attempted using Salmonella carrying a eukaryotic expression plasmid encoding HIV *env* (Shata et al. 2001). Again, oral administration of the recombinant bacteria led to the activation of antigen-specific CTL responses both in systemic and mucosal lymphoid tissue. Furthermore, Salmonella-mediated oral DNA delivery was applied for the vaccination against fungi and parasites as well as for the treatment of monogenic defects (reviewed by Loessner and Weiss 2004).

Salmonella strains have also been used for the delivery of plasmid DNA encoding tumor antigens. In the first instance, “model” tumor antigens such as β -galactosidase were encoded in eukaryotic expression plasmids such that the oral application of bacteria carrying these plasmids conferred protection against challenges with tumor cells expressing the relevant model antigen. For example, strain SL7207 transformed with plasmid pCMV β was shown to induce protection against a fibrosarcoma cell line that stably expressed *lacZ* (Paglia et al. 1998). The next studies assessed the efficacy of Salmonella to transfer autologous tumor antigens, which were expressed by the tumor itself but also by normal tissues. In this case, successful protection was observed with transgenes such as melanoma-associated antigen gp100 fused to the invariant chain and epitopes of gp100 and melanoma-associated antigen TRP2 fused to ubiquitin (Xiang et al. 2000, Weth et al. 2001). This suggested that Salmonella-mediated DNA vaccination can

overcome immunological tolerance against self antigens, maybe due to bacterial danger signals resulting in enhanced stimulation of the immune system. Moreover, tumor-targeted coadministration of IL-2 further enhanced the protective effect of the immunization (Xiang et al. 2000). Alternatively, cotransformation of a second plasmid encoding secretory IL-18, a cytokine known to suppress angiogenesis and to stimulate INF- γ production, resulted in a stronger antitumor effect in a murine breast cancer model than vaccination with *Salmonella* carrying only the tumor antigen plasmid (Luo et al. 2003).

To circumvent the problem of targeting genetically unstable tumor cells, an interesting novel approach has been used to target stable, proliferating endothelial cells in the tumor vasculature in order to inhibit angiogenesis. Strong cellular immune responses were elicited against these cells by vaccinating with *Salmonella* carrying a plasmid containing the vascular-endothelial growth factor receptor 2 (Niethammer et al. 2002). The study showed that protection against tumor challenge and reduced growth of metastases in a therapeutic setting could be achieved. Furthermore, this effect was enhanced, when the plasmid additionally encoded the IL-12 gene (Feng et al. 2005).

Several prerequisites have to be fulfilled in order to efficiently transfer DNA to host cells. The *Salmonella* carrier has to stably maintain the expression plasmid preferably in a high amount until invasion of the host cell takes place. Thereafter, the plasmids have to be released from the phagocytic compartment into the cytosol after lysis of the carrier strain, probably via leakage from host cell phagosomes (Schoen et al. 2004). The transfer of plasmid DNA from the bacteria into the cytosol and nucleus is still puzzling. Notably, cell specificity has been observed in *Salmonella*'s ability to mediate DNA transfer *in vitro*. *Salmonella* was found to transfect primary macrophages and DC (Darji et al. 1997, Dietrich et al. 2001b), whereas transfer into established cell lines was rather inefficient (Darji et al. 1997, Grillot-Courvalin et al. 2002). Thus, maybe host cell-specific pathways are exploited by the bacteria for the transfer of plasmid DNA into the cytosol (Weiss 2003). Finally, once released from the carrier, the plasmid has to enter the nuclear compartment for transcription. Therefore, beside inefficient release from the phagosome and detrimental effects of cytosolic as well as bacterial nucleases, this import constitutes an important obstacle in the transfection process. In summary, these different parts of the DNA transfer constitute starting points for the improvement of *Salmonella* as delivery system of DNA (see section 1.4).

1.3.4 **Salmonella in cancer treatment**

Besides using oral Salmonella-mediated DNA vaccination in tumor models, their capacity to preferentially target and replicate in tumor tissue has also been used in tumor therapy. This capacity was first demonstrated in 1997, when injected auxotrophic Salmonella were shown to specifically accumulate in the malignant tissue of tumor-bearing mice (Pawelek et al. 1997). The ratio of bacteria in the tumor to bacteria in normal tissues ranged between 250:1 and 9000:1 and this accumulation was accompanied by retarded tumor growth. Other studies have confirmed these findings and shown that attenuated Salmonella have bacteriolytic activity (Saltzman 2005). The precise molecular mechanisms for this anti-tumor effect are not known, but since SPI-1 mutant bacteria retain antitumor activity, it was suggested that invasion of the cancer cells seems not to be involved (Pawelek et al. 2002). In contrast, another group reported that infected tumor cells present antigens of bacterial origin and become targets for Salmonella-specific T cells (Avogadri et al. 2005). Additionally, massive recruitment of innate and adaptive effector cells at the site of infection and Salmonella-induced cross-presentation of tumor antigens were proposed to contribute to the antitumor activity. However, to amplify this effect, strains capable of delivering therapeutic molecules such as the herpes simplex thymidine kinase protein (Pawelek et al. 1997), endostatin (Lee et al. 2004) and thrombospondin-1 (Lee et al. 2005) have been produced.

A safety barrier for the utilization bacteria as systemically administered anticancer agents in humans is that they often massively stimulate TNF- α induction, which could lead to a cytokine cascade responsible for septic shock (Clairmont et al. 2000). In gram-negative bacteria this effect is mediated by lipid A, a component of the bacterial cell wall. By disrupting the *msbB* gene encoding myristil transferase, which is involved in the synthesis of this lipid, TNF- α induction could be reduced without losing the tumor-targeting and tumor-inhibiting properties (Low et al. 1999). VNP20009, a safe attenuated strain of *S. typhimurium* was generated that harbors deletions in the *purI* gene and *msbB* gene and remains susceptible to antibiotics (Clairmont et al. 2000). In initial clinical trials, this strain showed tumor colonization but no tumor regression was observed (Toso et al. 2002), even when a pro-drug converting enzyme was expressed (Nemunaitis et al. 2003).

Both bacterial and tumor-related factors have been implicated for the preferential accumulation of Salmonella in tumors. Within the tumor, areas of necrosis and apoptosis

may provide additional nutrients such as purines that are required by the auxotrophic strain (Mengesha et al. 2006). Likewise, *Salmonella* may exploit the hypoxic microenvironment (Anderson et al. 2006). Chemoattractive compounds produced by quiescent tumor cells have also been shown to contribute to the preferential accumulation (Kasinskas and Forbes 2006). Moreover, the tumor may provide an immunosuppressive environment, which protects bacteria from effectors of the immune system (Low et al. 1999). However, following the administration of *Salmonella* to tumor-bearing animals, also normal tissues are colonized, albeit transiently and to a lesser extent. In case of constitutive expression of therapeutic genes this might cause adverse side effects. Thus, it would be desirable to use regulated promoters that can be turned on either specifically in tumors or at certain time points when the bacteria have been cleared from normal tissues.

1.4 Improvement of *Salmonella* as delivery system in vaccination and tumor therapy

The development of *Salmonella* as delivery vector for therapeutic and prophylactic molecules has faced several problems, which resulted in vaccine failure or inefficient tumor therapy. However, progress in recombinant DNA technology and in the understanding of *Salmonella* pathogenicity as well as the generation of immune responses have provided solutions at least to some of these problems. Thus, a number of strategies hold promise for the improvement of the bacterial carrier and/or the properties of the cargo.

1.4.1 Attenuation of the bacterial carrier

Besides intrinsic properties of the antigen, the background of the carrier strain and the type of mutation selected to achieve attenuation critically affect the extent and quality of elicited immune responses (Dunstan et al. 1998, Valentine et al. 1998). Depending on the attenuating mutation specific types of immune responses can be stimulated (Medina et al. 1999). For example, a *Salmonella* PhoP mutant promotes potent innate immune responses of macrophages that are sufficient for host defense (VanCott et al. 1998). This mutant is more efficiently processed by macrophages *in vitro* than wild-type bacteria (Wick et al. 1995). In contrast, administration of an *aroA* attenuated strain elicits stronger antibody and T-helper cell responses, wherein Th1 T cells are required for clearance of the bacteria (VanCott et al. 1998). Thus, the type of immune response induced against the antigen can be already influenced by selecting an appropriate attenuation of the strain. Alternatively,

the type of mutation can also affect the ability of *Salmonella* to transfer the cargo. *Salmonella* with a *sifA* deletion can not preserve the SCV and have been shown to be more efficient in delivering eukaryotic expression plasmids *in vitro* than the wild-type counterpart (Michael et al. 2004, Petrovska et al. 2004). Moreover, increased specificity of the growth in tumor tissue could be recently achieved by creating Leu/Arg-dependent auxotrophic *Salmonella* mutants (Zhao et al. 2005, Zhao et al. 2006). These mutants were cleared from normal tissue even in immunodeficient mice, whereas the tumors were still colonized. Moreover, *Salmonella* were found to be dispersed throughout the malignant tissue and not only confined to necrotic tumor areas.

1.4.2 Stability of the recombinant phenotype

The instability of recombinant plasmids within the bacterial carrier is a serious problem in *Salmonella*-mediated delivery of heterologous proteins and DNA. Plasmid replication and retention represent a high metabolic burden for the bacteria (Galen and Levine 2001), potentially compromising the growth rate, fitness, persistence and immunogenicity. Studies with *E. coli* have established that plasmid-bearing bacteria grow more slowly than plasmid-less bacteria (McDermott et al. 1993). During biotechnological production steps, antibiotic selection keeps the pressure in favor of plasmid retention, but high-copy plasmids are often unstable *in vivo* leading to segregational loss of the foreign antigenic determinant and consequently to suboptimal vaccination or therapy (Spreng and Viret 2005). One possible solution of this problem is to integrate heterologous antigen-encoding genes into the chromosome of the *Salmonella* carrier. This approach increases the stability of the expression cassette but the amount of antigen may be insufficient to generate an efficient immune response (Hone et al. 1988, Strugnell et al. 1990, Cardenas and Clements 1993). Therefore, for ease of manipulation and copy number effects, plasmids are preferable which are stable *in vivo* and possess a copy number sufficient for the induction of an immune response without compromising fitness of the vector.

Plasmid-intrinsic properties, such as origin of replication, copy number, size and complexity can affect the plasmid retention by the bacterial carrier. For example, with increasing size of the plasmid, its stability decreases (Galen and Levine 2001). In addition, a relationship between plasmid stability and the copy number has been observed *in vitro* and *in vivo* (Dunstan et al. 2003). In this study, high-copy number prokaryotic expression plasmids were rapidly lost from the bacteria, whereas a reduction in copy number reduces

metabolic burden and consequently enhances plasmid retention. Moreover, the level of antigen-specific antibody induced by *Salmonella* directly correlated with the stability of the expression plasmid. Similarly, the effect of copy number was addressed in *Salmonella*-mediated delivery of DNA vaccines (Bauer et al. 2005). By exchanging the high-copy number pUC origin of replication of plasmid pCMV β with different low-copy number replicons (pMB1, p15A and pSC101) the plasmid stability could be increased both *in vitro* and *in vivo*. This increased stability of the eukaryotic expression plasmids resulted in an enhancement of antigen-specific CD4⁺ and CD8⁺ T cell and antibody responses even after a single oral immunization. On the other hand, the copy number should be as high as possible, since a very low-copy number plasmid induced weaker immune responses, despite being stably retained in *Salmonella*. Therefore, strategies that enhance antigen expression *in vivo*, e.g. a system for inducible amplification of copy numbers, have to be considered (Fig. 1.6). Such an approach allows maintenance of the plasmid copy number at low levels during culture of the vaccine strain. At a certain time point, e.g. upon entry of target cells, the plasmid copy number is increased. This strategy minimizes the metabolic burden of the bacteria during the invasion phase and is therefore suited for the improvement of *Salmonella* as carrier for therapeutic and prophylactic molecules.

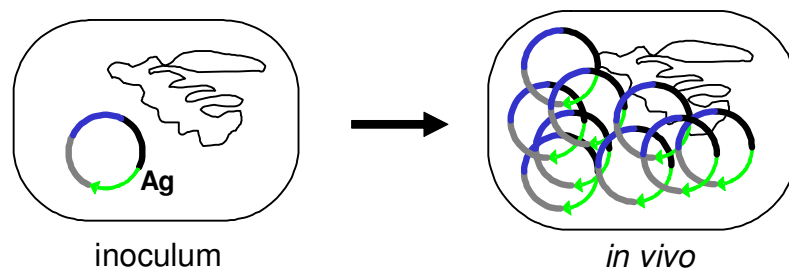


Fig. 1.6: Strategy for inducible plasmid amplification in a *Salmonella* vaccine strain. The plasmid is amplified starting from a minimal number in the inoculum to a high-copy number *in vivo*. Thus, sufficient plasmid DNA and antigen is provided.

As alternative to antibiotic resistance markers whose selective pressure is absent *in vivo*, balanced lethal stabilization systems have been developed (Spreng and Viret 2005). These systems are based on expression plasmids, which encode a gene that complements a defect of the carrier strain *in trans*. The *asd* system is a classic example for that approach (Nakayama 1988, Galan et al. 1990, Tacket et al. 1997, Nayak et al. 1998). The deletion of

the chromosomal *asd* gene that is required for cell wall synthesis results in absolute requirement for DAP. Since DAP is not found in mammalian tissue, only *Salmonella* carrying a plasmid that complements this defect are able to survive in the host. This does not solve the problem of general instable plasmids per se, but overgrowth of plasmid-less bacteria is prevented. Moreover, it abrogates the need for antibiotic resistance genes as selective markers. The criteria for live vaccines by the Food and Drug Administration (FDA) prohibit the use of resistance genes, since they may spread to pathogenic organisms in the environment (Garmory et al. 2002). Besides the *asd* system, balanced lethal systems based on the *thyA* gene have been explored (Morona et al. 1991, Yoshikawa et al. 1995). The *thyA* gene encodes the enzyme thymidilate synthase, which plays a key role in the *de novo* DNA synthesis. Mutants that lack the enzymatic activity are dependent on exogenous thymidine or thymine (Ross et al. 1990), thus undergoing “thymidineless death” without such nutrients (Ahmad et al. 1998).

1.4.3 Targeting of antigen delivery

Heterologous antigens that are expressed in the cytoplasm of the bacterial carrier are only accessible to the immune system after disintegration of the bacteria. In addition, conventional antigen expression might lower the genetic stability of the vaccine strain due to intrinsic toxicity of the antigen or reduce the immunogenicity due to rapid degradation of the candidate antigen. To circumvent this problem, several systems for the secretion of antigens into the extracellular milieu or the expression of foreign proteins on the surface of the vector have been developed. The latter include insertion of the antigen into cell surface proteins of *Salmonella*, such as flagellar components (Newton et al. 1989) and outer membrane proteins (Isoda et al. 2007). Furthermore, autotransporter secretion pathways of gram-negative bacteria have been employed for surface display of antigenic determinants in *Salmonella* vaccine strains (Kramer et al. 2003). Autotransporters are expressed as a single polypeptide chain that provides all functions necessary to translocate a passenger antigen and to display it on the bacterial cell surface. Consequently, antigen expression via the MHC class II pathway occurs when the antigen is displayed by *Salmonella* residing in the phagosome. Fusion of the antigen to the autotransporter domain of the *E. coli* adhesion involved in diffuse adherence AIDA-I resulted in high genetic stability of the vaccine strain and in a pronounced Th1-biased MHC class II restricted cellular immune responses (Kramer et al. 2003). Foreign antigens may also be secreted by the use of signal sequences. A popular system is the hemolysin A (HlyA) export-expression system of uropathogenic

E. coli (Gentshev et al. 2002). Heterologous antigens are linked at their carboxyl terminus to the secretion signal of HlyA and secreted by the hemolysin type I secretion apparatus. A variety of antigens of bacterial, viral and parasitic origin have been expressed and secreted in attenuated *Salmonella* spp., thereby triggering humoral and/or cell-mediated immune responses. In the murine listeriosis model a superior protective efficacy of secreted versus somatic LLO expression was observed (Hess et al. 1996).

Antigen secretion by a phagosomal vaccine carrier like *Salmonella* preferentially induces CD4⁺ T cell responses. In contrast, direction of antigens to the cytosol will consequently enhance the capacity of the vector to induce CTL responses. For example, *Salmonella* were modified to secrete active LLO fused to the HlyA secretion signal, which resulted in lysis of the phagosomal membrane and access to the host cell cytosol (Dietrich et al. 2001a). Access to the cytosol was found to enhance presentation of passenger proteins *in vitro* (Catic et al. 1999). In the same study, such modified *Salmonella* were also used to deliver a eukaryotic expression plasmid into macrophages. This phagosomal escape strategy was found to enhance DNA delivery. Recently, the *Salmonella* type III secretion system has been used as delivery means for heterologous proteins. It offers the advantage to directly inject the proteins from the bacteria into the host cell cytosol (Russmann et al. 1998). This can be achieved by fusing antigens to effector proteins translocated by TTSS (Husseiny et al. 2007). Alternatively, it was shown that effector protein YopE of the TTSS of *Yersinia* spp. can also mediate the translocation of fusion proteins by the SPI-1 TTSS (Russmann et al. 2001).

1.4.4 Lysis of the bacterial carrier

The delivery of antigens or DNA vaccines requires liberation of the molecules after invasion of the host. Besides the use of secretion systems that encounter limitations regarding size, structure and amount of the cargo, an alternative for that purpose would be release upon rupture of the cell wall. The introduction of an appropriate attenuation into the carrier strain is a simple method to achieve the lysis of the carrier. For example, *Salmonella* Δ *asd* quickly undergo lysis in environments lacking DAP, which leads to the release of protein, such as active LLO, and DNA (Loessner et al. 2006). Since *Salmonella* Δ *asd* might be overattenuated *in vivo* regarding host colonization, conditional mutant strains might have to be constructed for therapeutic or prophylactic purposes. In an alternative effort to produce *Salmonella* strains for improved DNA vaccine delivery, a

system for the programmed lysis of the bacteria has been described (Jain and Mekalanos 2000). The S and R gene products of phage lambda were used to lyse *S. typhimurium* under control of the L-arabinose inducible promoter P_{BAD} *in vitro*. Additionally, it was shown that the release of plasmid DNA from bacteria could be augmented by the use of mutants lacking nuclease activity.

1.4.5 Multivalent Salmonella vaccines

The ability of attenuated Salmonella to elicit a broad spectrum of immune responses against a wide range of pathogens is well established (Detmer and Glenting 2006). Using the variety of expression systems developed so far, it should be possible to engineer multivalent Salmonella vaccines that express antigens derived from several pathogens (Atkins et al. 2006). For example, the use of compatible eukaryotic expression plasmids in the same carrier conferred immunity to the different plasmid derived antigens (Bauer et al. 2005). This strategy is also applicable for the coadministration of plasmids encoding cytokines or costimulatory molecules. In addition it might be possible to simultaneously express chromosomally integrated heterologous antigens and plasmid-encoded antigens or to transfer a DNA plasmid at the same time (Garmory et al. 2002). In order to develop such bivalent strains the use of appropriate regulatory elements might be of great importance.

1.4.6 Regulatory elements

1.4.6.1 Choice of promoter

The promoter controlling expression of the antigen determines the amount of antigen produced. In the case of DNA vaccines, virally derived promoters, especially the CMV immediate early promoter, have been extensively used to drive antigen expression in the eukaryotic host cell. They ensure strong expression *in vivo* compared to other eukaryotic promoters (Norman et al. 1997). On the other hand, it was demonstrated that virally derived promoters are downregulated by cytokines such as IFN- γ or TNF- α (Qin et al. 1997), which are induced during Salmonella-mediated DNA vaccination. Alternatively, strong promoters of housekeeping genes or promoters that are active in APC, e.g. the DC-specific CD11c promoter, can be applied for genetic immunization.

In case of protein delivery, a strong promoter increases the bacterial metabolic burden, which often results in the instability of expression plasmids. Recently, *Salmonella* vectors with regulated instead of constitutive antigen expression have been employed in order to decrease the toxicity to the carrier and the metabolic burden during early phases of host colonization (Chatfield et al. 1992a, Bullifent et al. 2000, Huang et al. 2000, McKelvie et al. 2004, Stratford et al. 2005, Husseiny and Hensel 2005). In these studies the antigen is expressed using *in vivo* inducible (IVI) promoters that are upregulated when the vector enters the APC of the host thereby receiving certain environmental stimuli. One example is the *nirB* promoter, which is induced in anaerobic environments and by entry of *Salmonella* into cells. (Chatfield et al. 1992a). Using this promoter to express an immunogenic fragment of tetanus toxin, superior immune responses have been observed in comparison to the strong constitutive *tac* promoter, which could be attributed to higher plasmid stability. The macrophage-inducible *pagC* promoter, controlled by the two-component regulatory system PhoP/PhoQ, has also been shown to provide stable, high-level expression of passenger antigens in *Salmonella* (Dunstan et al. 1999, Bullifent et al. 2000, Arnold et al. 2004). A comparison of seven different promoters in *Salmonella* used flow cytometry to quantify *in vivo* antigen levels and to simultaneously monitor the early steps of antigen-specific T cell responses in mice (Bumann 2001b). The study demonstrated that *in vivo* expression levels for an antigen can be rationally selected by choosing from a set of promoters with defined properties, perhaps enabling the fine-tuning and timing of expression to direct specific immune responses. Additionally, it pointed out that the very high *in vitro* activity of the strong constitutive *tac* promoter led to impaired early colonization of the recombinant *S. typhimurium* strain, which consequently reduced the efficacy in generating an immune response. Most of the IVI promoters, which have been tested for antigen expression, are part of operons encoding for *Salmonella* virulence genes, thereby taking advantage of the spatiotemporal expression patterns of these genes (Brown et al. 2005). In this context, a possible limitation for promoters of genes located on SPI-1 might be their downregulation after uptake by host cells. In contrast, effector proteins of SPI-2 are specifically induced by intracellular *Salmonella* after entering lymphatic tissues (Bumann 2002), highlighting their suitability for *in vivo* inducible antigen expression for vaccination.

Following the administration of attenuated *Salmonella* to tumor-bearing animals, the bacteria colonize also normal tissues to a certain extent. Therefore, the use of a constitutive

promoter to drive therapeutic genes of interest might cause undesired side-effects. One way to address this problem is to exploit promoters, which are specifically activated in the tumor microenvironment. The lower oxygen levels compared to normal tissue are a characteristic feature found in solid tumors. Recently, a prokaryotic *Salmonella* gene delivery vector was genetically engineered to spatially control gene expression using the hypoxia-inducible promoter HIP-1 (Mengesha et al. 2006). Using this promoter, reporter gene expression was confined to tumors made hypoxic. In another study, bacteria were engineered to invade cancer cells *in vitro* under control of certain environmental stimuli, that is hypoxia, cell density and L-arabinose (Anderson et al. 2006). This strategy could be applied to processes other than invasion, such as the release of a therapeutic protein. Moreover, stimuli that are not intrinsic to the host but can be administered at will provide a possibility to temporally and/or spatially control gene expression in order to increase specificity of tumor therapy.

1.4.6.2 Remote control of bacterial carriers

A range of bacterial promoters have been identified, which respond to various stimuli, such as particular substrates, temperature, hypoxia, ionic strength, radiation, pH, cation levels and other environmental conditions (Cases and de Lorenzo 1998, Lucas and Lee 2000). To deliberately modify gene expression of bacterial carriers *in vivo* at a defined time, e.g. for tumor therapy and vaccination, a combination of a suitable promoter and extrinsic inducer is needed. Thus far, only one bacterial system has been described to spatially and temporally control expression within the host. Nuyts *et al.* recently identified two clostridial promoters, *recA* and *recN*, which could be induced by ionizing radiation (Nuyts et al. 2001b). Using this approach, the expression of therapeutic genes by *Clostridia* could be further enhanced by simultaneous application of radiotherapy (Nuyts et al. 2001a). However, the application of irradiation as inducer is hampered since it also results in damage of normal tissue.

An ideal inducer to control bacterial carriers for therapeutic and prophylactic purposes should fulfill several prerequisites, some of which are: it should be i) nontoxic and non-immunogenic to the host, ii) able to be administered repeatedly by established routes (e.g. orally and intravenously) in a wide range of doses, iii) able to reach the bacterial niches *in vivo* iv) readily available at low cost. Additionally, it is desirable that the activity of the responding promoter is very tightly controlled. In this context, one promising expression

system is based on the L-arabinose-inducible promoter P_{BAD} (Guzman et al. 1995). The P_{BAD} promoter from the arabinose operon of *E. coli* offers high-level expression and very inexpensive induction with the sugar L-arabinose. In *E. coli*, P_{BAD} controls the transcription of the genes *araB* (ribulokinase), *araA* (isomerase) and *araD* (epimerase), whose products mediate the metabolic conversion of the sugar L-arabinose (reviewed by Schleif 2003). This promoter is regulated by the protein AraC, which negatively controls its own transcription. In the absence of L-arabinose, the dimeric AraC protein binds to the initiator region I1 and the operator region O2, thus leading to the formation of a DNA loop upstream of P_{BAD} that represses the promoter. In the presence of L-arabinose, AraC binds the sugar, changes its conformation and releases the DNA loop. In this state, it binds to the initiator regions I1 and I2 upstream of P_{BAD} and stimulates binding of the activating complex formed by cyclic AMP (cAMP) and cAMP receptor protein as well as binding of the RNA polymerase, thus efficiently starting transcription. In summary, P_{BAD} is very tightly regulated, since the uninduced levels can be reduced even further by growth in the presence of glucose. Glucose reduces levels of cAMP, consequently lowering expression of the catabolite-repressed P_{BAD} promoter (Miyada et al. 1984). Therefore, this system is widely used to control expression in bacterial cultures. As the inducer L-arabinose is also in wide use for dietary purposes as well as a formulation agent for pharmaceutical preparations, this system is an attractive candidate for *in vivo* remote control of Salmonella.

1.5 Aims of this work

Live attenuated Salmonella are explored as vectors for the delivery of heterologous antigens and DNA vaccines as well as for tumor therapy. Efficacy of vaccination often corresponds to the amount of antigen presented or the DNA dose delivered. However, antigen expression at high levels and maintenance of the plasmid DNA at high-copy numbers seriously weakens the bacteria resulting in a decreased potential of the bacteria in terms of invasiveness, fitness and immunogenicity. The aim of this work was the development of novel Salmonella strains with amplifiable expression plasmids.

In the first part of the thesis, a strategy should be established to amplify expression plasmids under control of Salmonella-specific *in vivo* inducible promoters. The vaccine strain should maintain the expression plasmid in a single copy state in the inoculum and initiate amplification to a high-copy number upon induction of the *in vivo* inducible promoters after bacterial administration. Novel strains developed with these promoters

should be evaluated by using reporter plasmids whose amplification could be followed *in vitro* and *in vivo*. As a second step, the inducible system for plasmid amplification should be further improved by enhancing expression due to simultaneous control of plasmid copy number and protein transcription for the delivery of passenger antigens. Finally, the novel vaccine strains should be tested in the mouse model regarding colonization capabilities and stability of the vector system. Additionally, their immunogenicity should be tested using the model antigens ovalbumin and listeriolysin.

Complementary, plasmid amplification should be developed to be inducible at any time *in vivo*. To remotely control the bacterial carrier in this aspect, the amplifiable system had to be linked with the L-arabinose inducible P_{BAD} promoter. The ability of the sugar L-arabinose to induce plasmid amplification in medium, in cell culture and *in vivo* had to be evaluated. Subsequently, L-arabinose-mediated plasmid amplification and simultaneous protein expression should be characterized in tumor-bearing mice.

2 Material and methods

2.1 Bacterial strains and growth conditions

The *Escherichia coli* (*E. coli*) strains DH5 α and Top10 (Invitrogen, Groningen, The Netherlands) were used for general cloning. The auxotrophic *Salmonella enterica* Serovar Typhimurium strain SL7207 (*hisG46*, $\Delta 407[aroA544::Tn10]$, kindly provided by Dr. B. A. D. Stocker, (Stanford, CA, USA) was used as plasmid carrier for the *in vitro* and *in vivo* studies. *E. coli* strain DH5 α λ pir was used for cloning of plasmids with the λ pir-dependent R6K origin of replication and *E. coli* strain SM10 λ pir was used for Tn7-mediated transposition in SL7207. *E. coli* and SL7207 were routinely grown in Luria Bertani (LB) broth or on LB-agar plates (Sambrook et al. 1989) at 37°C or 30°C (DH5 α λ pir and SM10 λ pir). SL7207 was grown in induction medium (IM) when indicated. IM is M9 (Sambrook et al. 1989) without CaCl₂ supplemented with 100 μ M MgSO₄, 40 μ g ml⁻¹ histidine, 40 μ g ml⁻¹ phenylalanine, 40 μ g ml⁻¹ tryptophane, 40 μ g ml⁻¹ tyrosine, 10 μ g ml⁻¹ para-aminobenzoic acid, 10 μ g ml⁻¹ 2,3-dihydroxybenzoate; the pH was adjusted to 5,5 with concentrated HCl. Antibiotics were added at the following concentrations where required: 30 μ g ml⁻¹ streptomycin; 12,5 μ g ml⁻¹ chloramphenicol; 30 μ g ml⁻¹; kanamycin; 100 μ g ml⁻¹ ampicillin. *Listeria monocytogenes* EGDe for *in vivo* cytotoxicity assays was grown in Brain Heart Infusion (BHI) broth or on BHI-agar plates (Difco, Detroit, MI) at 37°C (Leimeister-Wachter and Chakraborty 1989).

2.2 Cell lines

TS/A (Nanni et al. 1983), CT26 (ATCC CRL-2638) and J774-A.1 (ATCC TIB67) were cultured in IMDM (Gibco BRL, Eggenstein, Germany) supplemented with 10% heat-inactivated FCS (Integro, Zaandam, The Netherlands), 250 mM β -mercaptoethanol (Serva, Heidelberg, Germany) and 100 μ g ml⁻¹ penicillin/streptomycin (Biochrom, Berlin, Germany). Cells were cultured at 37°C and 5% CO₂ in a humidified atmosphere.

2.3 Mouse strains

Female or male BALB/c (H-2^d) mice were purchased from Harlan Winkelmann (Borchen, Germany) and used at age 8-12 weeks for all experiments. Transgenic DO11.10 mice were

bred and maintained in a specific pathogen-free animal facility at the HZI or obtained from the Bundesinstitut für Risikobewertung (BfR), Berlin.

2.4 Oligonucleotides

Primer	Nucleotide sequence (5'-3'-direction)
AEN22	GGATATCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATAT ACCATGAACGGACGTTTGA
AEN23	GGATATCAAGCTTCTGCAGGTCGACCTAGC GTTTGCAATGC
AEN32	AAGGCACCGACGTTGACCA
AEN43	ATGTTGGACGAGTCGGAATC
AEN61	GATATCCATATGGAACAAAACTTATTTCTGAAGAAGATCTGAT GCCTAAGACGCCAATCGAAAAG
AEN63	GATATCCTGCAGGGATCCTTAGTCGATGATTGAACTTCAT

Primers were ordered from Operon (Köln, Germany).

2.5 Plasmids

2.5.1 Outgoing plasmids for cloning

Plasmid	Source/reference
pETcoco-1	Expression plasmid, which is amplifiable from 1-2 to 20-50 copies/cell under control of P_{BAD} (Novagen, Merck KGaA, Darmstadt, Germany)
p3.6B	Expression plasmid derived from pGFP_ova (Bumann 2002), contains P_{sifA} (kindly provided by Dr. Dirk Bumann)

Plasmid	Source/reference
p2.2A	Expression plasmid derived from pGFP_ova (Bumann 2002), contains P_{phoN} (kindly provided by Dr. Dirk Bumann)
pCLII.3A	Expression plasmid derived from pGFP_ova (Bumann 2002), contains $P_{stm1630}$ (kindly provided by Dr. Dirk Bumann)
pHL49	GFP-expression plasmid (Cormack et al. 1996) with the β -lactamase promoter P_{bla} (H. Löbner, unpublished)
pHL230	Plasmid derived from pET-11 (Novagen), contains a T7 terminator sequence (H. Löbner, unpublished)
pHL222	Expression plasmid for firefly luciferase derived from pGL3-basic (Promega, Mannheim, Germany) with the β -lactamase promoter P_{bla} (H. Löbner, unpublished)
pCMV β m4A	Eukaryotic expression plasmid with pSC101 origin of replication (Bauer et al. 2005)
pHL259	L-arabinose inducible luciferase expression plasmid (Loessner et al. 2007)
pCMV β m2A	Eukaryotic expression plasmid with pMB1 origin of replication (Bauer et al. 2005)
pCMV β m3A	Eukaryotic expression plasmid with p15A origin of replication (Bauer et al. 2005)
pUX-BF13	Tn7-helper plasmid (Bao et al. 1991)
pCMVhly	Eukaryotic expression plasmid for LLO (Darji et al. 1997)

Plasmid	Source/reference
pSLE1f	Carrier plasmid for Tn7-mediated transposition (S. Leschner, unpublished), derivative of pUX-BF5 (Bao et al. 1991)
pHL260a	Carrier plasmid for Tn7-mediated transposition, contains <i>gene E</i> under control of P_{BAD} (Loessner et al. 2007)
pHL238	L-arabinose inducible GFP expression plasmid (Loessner et al. 2007)
pSL1	Expression plasmid with <i>luxCDABE</i> operon (Winson et al. 1998), derivative of pSMART-LCKan (Lucigen, Middleton, USA)

2.5.2 Reporter plasmids for L-arabinose inducible amplification

A $P_{bla-gfp_ova}$ reporter construct was made by cloning a *SalI/XbaI* fragment of pHL49 containing the promoter of the ampicillin resistance gene (*bla*) into the *SalI/XbaI* fragment of the vector p3.6B. The resulting plasmid was digested with *NaeI* and *HindIII* to introduce the $P_{bla-gfp_ova}$ reporter construct into the *HpaI/HindIII* fragment of the expression vector pETcoco-1, yielding plasmid pAEN7. A T7 terminator sequence was introduced into pAEN7 by ligating a blunt-ended *EcoRV* fragment of plasmid pHL230 with the *HindIII*-digested, blunt-ended vector, yielding plasmid pAEN9 (Fig. 2.1).

To create plasmid pAEN17 with *gfp* instead of *gfp_ova*, the $P_{bla-gfp}$ fragment was removed from pHL49 by digestion with *NotI/HindIII*, followed by Klenow treatment to generate blunt-ends and inserted into the *NotI* digested blunt-ended vector pAEN9. Similarly, plasmid pAEN38 contains *luc* instead of *gfp_ova* as reporter gene. A $P_{bla-luc}$ fragment was isolated from plasmid pHL222 by digestion with *EcoRI/NaeI*, blunt-ended and inserted into the *NotI* digested and blunt-ended vector pAEN9. The pSC101 origin of replication containing fragment from plasmid pCMVm4A was isolated by *BglIII/XbaI* digestion and subsequent Klenow treatment to generate blunt-ends. The vector pAEN38 was digested with *BglIII* and *NotI*, blunt-ended and the two fragments were ligated, resulting in plasmid pAEN59 (Fig. 2.2).

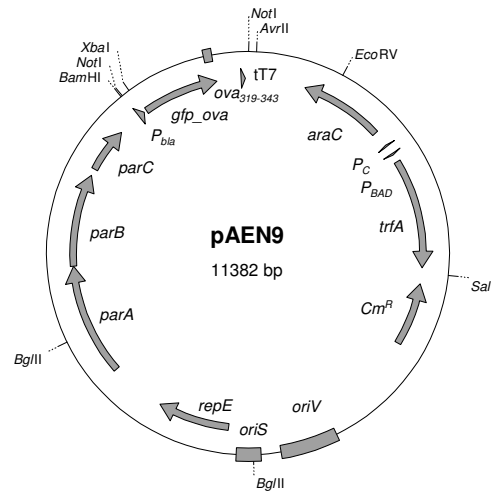


Fig. 2.1: Plasmid map of pAEN9. The *lacI* containing fragment of pETcoco-1 was replaced by *P_{bla}-gfp_ova* and a T7 terminator, see text for construction details.

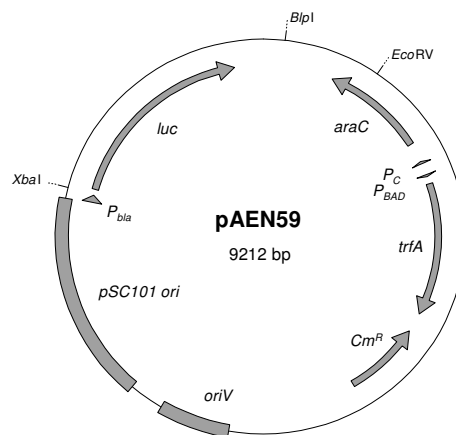


Fig. 2.2: Plasmid map of pAEN59. In contrast to plasmid pAEN38, this plasmid carries the pSC101 origin of replication to maintain the minimal-copy state.

The *P_{bla}-gfp_ova* fragment of pAEN9 was excised with *XbaI/EcoRV* and inserted into the *XbaI/EcoRV* sites of pAEN59. The resulting plasmid pAEN60 was then digested with *XbaI* and *BlnI* to exchange the reporter gene with the *P_{BAD}-luc* containing *XbaI/BlnI* fragment of pHL259, yielding pAEN64 (Fig. 2.3). The *P_{BAD}-trfA* cassette was isolated by digesting pAEN64 with *EspI/NdeI*, blunt-ending and subsequent ligation, yielding plasmid pAEN110.

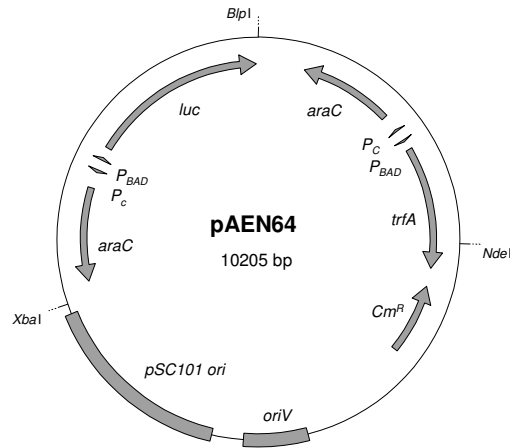


Fig. 2.3: Plasmid map of pAEN64. Plasmid pAEN110 is similar but not amplifiable via L-arabinose due to excision of P_{BAD} -*trfA*.

2.5.3 Reporter plasmids for *in vivo* inducible plasmid amplification

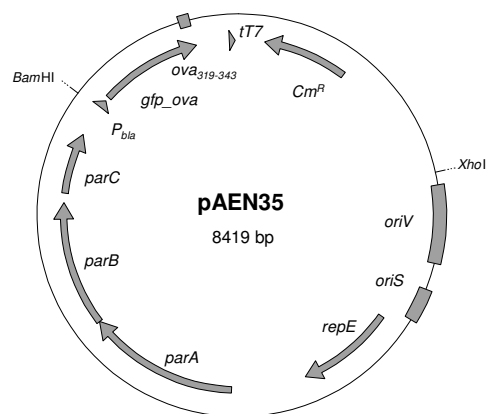


Fig. 2.4: Plasmid map of pAEN35. This plasmid is a derivative of pAEN9, which was digested with *AvrII* and *SalI* to remove the P_{BAD} -*trfA* cassette, treated with Klenow fragment and ligated.

Plasmid pAEN9 was digested with *SalI* and *BamHI*, blunt-ended and the fragment containing the F plasmid replicon, *oriV* and Cm^R was ligated with a blunt-ended P_{sifA} -*gfp_ova* fragment derived from p3.6B that was digested with *SalI/HindIII*. The resulting plasmid pAEN51 (Fig. 2.5) was used to introduce the pSC101 replicon by digesting the plasmid with *BglII* and *BspLU11I*, blunt-ending and ligation with the blunt-ended *BglII/XbaI* fragment of pCMV β m4A, yielding pAEN61 (Fig. 2.6).

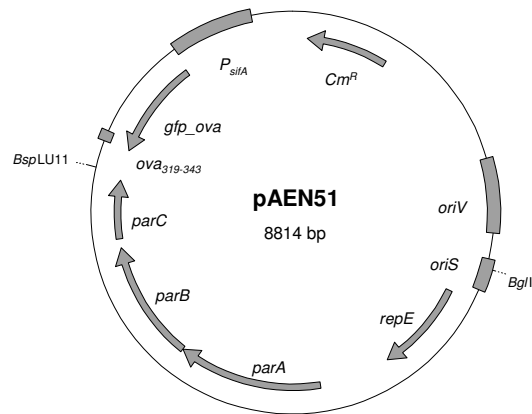


Fig. 2.5: Plasmid map of pAEN51. This plasmid is similar to pAEN35, but the P_{bla} - gfp_ova reporter was replaced with P_{sifA} - gfp_ova .

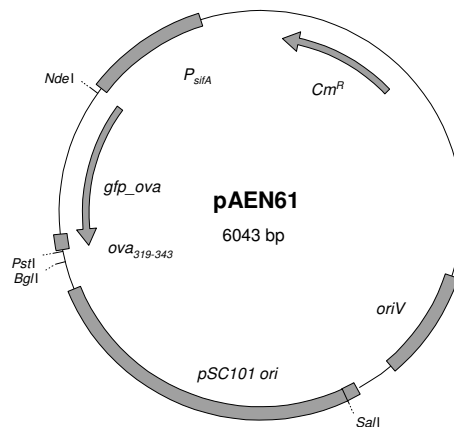


Fig. 2.6: Plasmid map of pAEN61. In contrast to plasmid pAEN51, this plasmid carries the pSC101 origin of replication to maintain the minimal-copy state.

Plasmid pAEN61 was used to introduce the pMB1 and p15A replicon by digesting the plasmid with *Bgl*II and *Sal*I, blunt-ending and ligation with the blunt-ended *Bgl*III/*Xba*I fragment of pCMV β m2A and pCMV β m3A, yielding pAEN108 and pAEN109, respectively.

To create a reporter plasmid for *in vivo* imaging, the coding sequence for the *luxCDABE* operon originating from *Photorhabdus luminescens* was obtained by restriction digestion of pSL1 with *Sna*BI and *Nco*I followed by blunt-ending. Then the coding sequence for

gfp_ova was removed from pAEN61 by digestion with *NdeI/BglI*, the vector was treated with T4 DNA polymerase and ligated with the *luxCDABE* operon.

2.5.4 Plasmids for Tn7-mediated transposition

Plasmid pSL1f was used to construct carrier plasmids for Tn7-mediated transposition in SL7207. pSLE1f carries a kanamycin resistance gene between the transposon ends Tn7L and Tn7R as well as a single *SalI* restriction site for the insertion of DNA fragments. To construct derivatives of this plasmid, the vector was opened with *SalI* and treated with Klenow to generate blunt-ends.

The P_{BAD} -*gfp* containing fragment of plasmid pHL238 was isolated by digestion with *PvuII/HindIII*, subsequently treated with Klenow and ligated with the vector pSLE1f. The resulting plasmid pAEN44 was used to generate a SL7207 strain that contained the reporter *gfp* under control of the L-arabinose inducible promoter P_{BAD} . Plasmid pAEN46 served as carrier plasmid for the insertion of a P_{BAD} -*trfA* cassette into the chromosome of SL7207. This cassette was derived from plasmid pAEN9 after digestion with *AvrII/SalI* and Klenow treatment and inserted into pSLE1f.

P_{IVT} -*gfp* reporter constructs were made using plasmid pHL49 by inserting the *SalI/NdeI* promoter fragments of p2.2A, pCLII.3A and p3.6b into the *SalI/NdeI* digested vector. The resulting plasmids were digested with *SalI* and *HindIII*, blunt-ended and ligated with *SalI* opened, blunt-ended pSLE1f yielding pAEN45.1 (P_{phoN} -*gfp*), pAEN45.2 ($P_{stm1630}$ -*gfp*) and pAEN45.3 (P_{sifA} -*gfp*). To construct transcriptional fusions of *in vivo* inducible promoters with *trfA*, the gene was amplified from pETcoco-1 by PCR using primers AEN22 and AEN23. The PCR product was digested with *XbaI* and *HindIII* and exchanged for *gfp_ova* in p2.2A, pCLII.3A and p3.6b to yield pAEN8.1-3. The plasmids were digested with *SalI* and the P_{IVT} -*trfA* fusions inserted into the unique *SalI* restriction site of pSLE1f yielding pAEN47.1(P_{phoN} -*trfA*), pAEN47.2 ($P_{stm1630}$ -*trfA*) and pAEN47.3 (P_{sifA} -*trfA*).

Carrier plasmid pAEN106 was made by inserting the P_{sifA} -*trfA* containing *SalI* fragment of pAEN47.3 into the unique *SalI* restriction site of pHL260a. Plasmid pAEN106 was used to generate a chromosomal integration derivative of SL7207, which contains P_{sifA} -*trfA* as well as *gene E* of bacteriophage Φ X174 under control of the P_{BAD} promoter.

2.5.5 Expression plasmids with listeriolysin O as antigen

To introduce another antigen instead of *ova*, a part of the *hly* gene (amino acids 49-361) was amplified by PCR using primers AEN61 and AEN63 and pCMVhly as template. Primer AEN61 contains a recognition sequence for *NdeI* and a myc-tag, primer AEN63 introduces the recognition sequence for *PstI* and *BamHI* as well as a stop codon. The PCR product was cloned into pCR2.1-TOPO (Invitrogen, Groningen, The Netherlands) and the insert was verified by sequencing. The insert was retrieved with *NdeI* and *PstI* and cloned into *NdeI/PstI* sites of pAEN61, yielding plasmid pAEN95 (Fig. 2.7). To replace the pSC101 replicon with the F plasmid replicon, plasmid pAEN95 was digested with *XhoI/BamHI* and the fragment containing the antigen and the resistance cassette was cloned into the *XhoI/BamHI* sites of pAEN35.

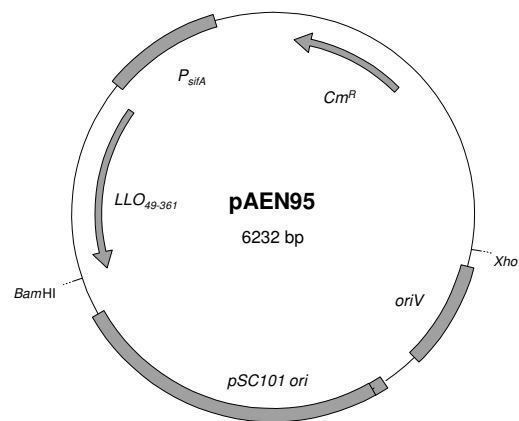


Fig. 2.7: Plasmid map of pAEN95. Plasmid pAEN95 is similar to pAEN61 but it contains *LLO₄₉₋₃₆₁* as antigen instead of *gfp_ova*. Likewise, pAEN98 carries *LLO₄₉₋₃₆₁* as antigen in contrast to pAEN51.

2.6 Chromosomal integration derivatives of SL7207

In this work, transcriptional fusions of inducible promoters with the reporter gene *gfp* and the replication protein TrfA were integrated into the chromosome of strain SL7207 by Tn7-mediated transposition (Fig. 2.8). The specific insertion occurs at the transcriptional inactive site *attTn7*, which is flanked by the bacterial genes *phoS* and *glmS*.

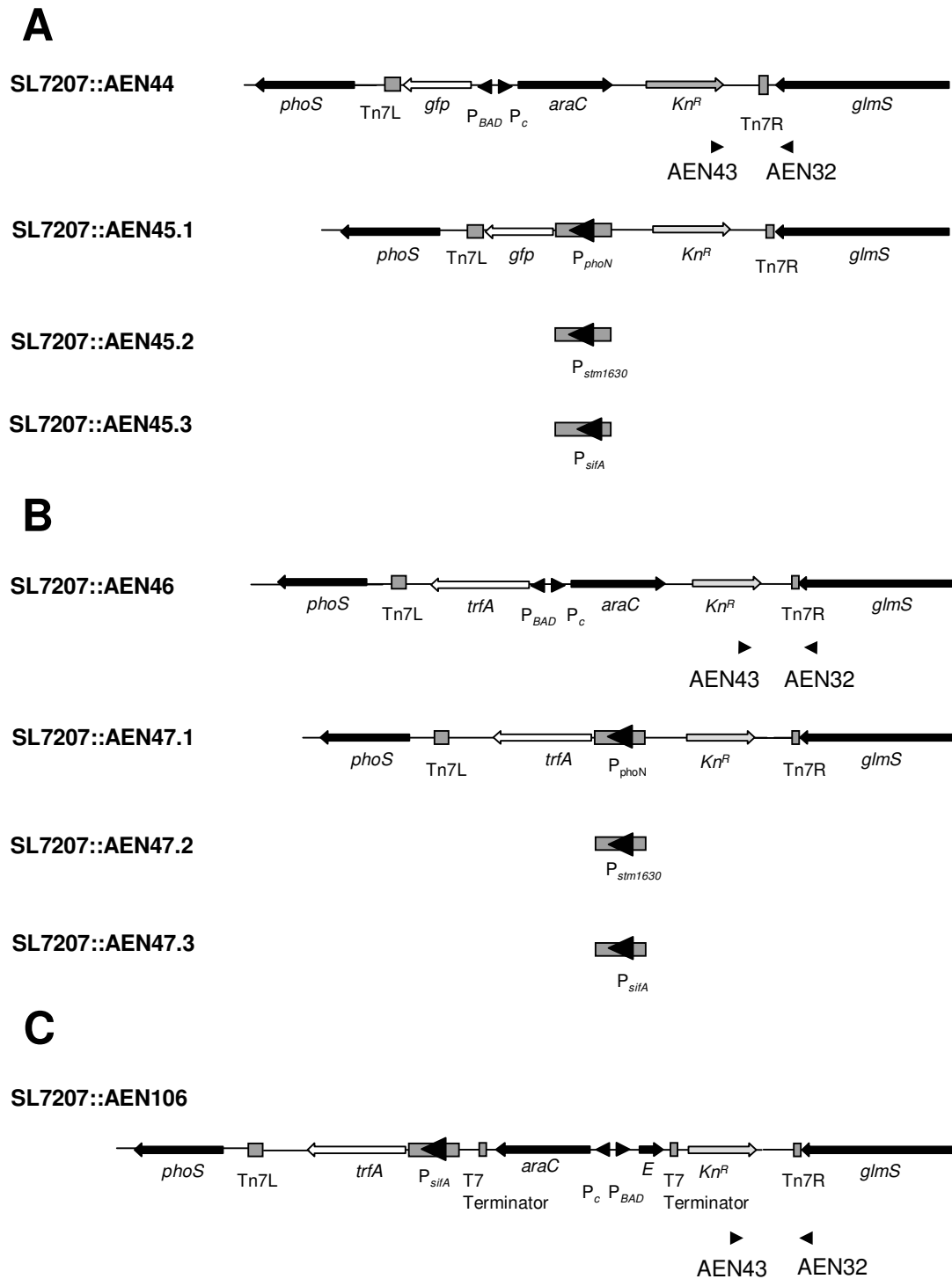


Fig. 2.8: Schematic display of the chromosomal integration derivatives of SL7207. Transcriptional fusions of *gfp* (A) and *trfA* (B) with different inducible promoters. (C) SL7207::AEN106 contains a fusion of P_{sifA} with *trfA* as well as *gene E* of bacteriophage Φ X174 under control of the P_{BAD} promoter. To check the correct chromosomal integration, a PCR with the primers AEN32/43 amplifying a 708 bp fragment between Kn^R and *glmS* was performed.

2.7 Molecular biological methods

2.7.1 Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate linearized, double-stranded DNA fragments for analytical and preparative purposes. This method is based on the size-dependent migration of DNA fragments in an electric field. The size of the fragments was estimated by comparison with marker DNA of known size, a mix of *Hind*III-digested λ -DNA and *Hae*III-digested p ϕ X174-DNA (MBI Fermentas, St. Leon-Rot, Germany). In this study, 0,8% agarose (Appligene, Heidelberg, Germany) was used in TAE buffer (Sambrook et al. 1989) and gels were run at 80-150 V. Gels were stained with ethidium bromide, visualized with UV light and documented.

2.7.2 Preparation of DNA

2.7.2.1 Isolation of DNA from agarose gels

DNA was extracted from agarose gels using the “QIAquick Gel Extraction Kit” (Qiagen, Hilden, Germany) according to the manufacturer’s instructions and subsequently eluted in 35 μ l H₂O.

2.7.2.2 Isolation of plasmid DNA

The isolation of plasmid DNA from bacteria was carried out in mini or midi plasmid preparation depending on the required amount. The “GFX Micro Plasmid Prep Kit” (Amersham Biosciences, Freiburg, Germany) and the “Qiagen Plasmid Midi Kit” (Qiagen, Hilden, Germany) were used to purify plasmids from *E. coli* strains. To isolate plasmids from *Salmonella* strains, the “Qiagen Plasmid Mini Kit” was employed.

2.7.2.3 Determination of DNA concentration

The DNA concentration was determined optically by measuring the extinction of a sample at 260 nm in a BioPhotometer (Eppendorf, Hamburg, Germany). Using a 1 cm cuvette, an OD₂₆₀ of 1 corresponds to a concentration of 50 μ g ml⁻¹. Alternatively, the concentration of linearized DNA was estimated by visual comparison of the band intensity with that of marker DNA containing defined quantities of DNA.

2.7.3 Molecular cloning

For analytical or preparative purposes, DNA was digested with appropriate restriction endonucleases of different manufacturers (MBI Fermentas, St. Leon-Roth, Germany; New England Biolabs, Frankfurt am Main, Germany; Roche Diagnostics, Mannheim, Germany). The digestion was carried out using optimal buffer and temperature conditions according to the manufacturer's instructions.

To ligate two DNA strands without compatible restriction sites, overhanging DNA ends were modified to generate blunt-ends. In case of 3' overhangs, 1 unit of T4 DNA polymerase (MBI Fermentas, St. Leon-Roth, Germany) and 2,5 µl 2 mM dNTP were added to a restriction digestion of 20 µl and incubated at room temperature for 10 min. To fill-in 5' overhangs, the same reaction was performed using 2 U Klenow fragment of *E. coli* DNA-polymerase I (MBI Fermentas, St. Leon-Roth, Germany) and incubating at 30°C for 15 min. Prior to ligation with blunt-ended DNA fragments, the vector DNA was dephosphorylated in order to increase the insertion frequency by avoiding self-ligation of the plasmid vector. The reaction was carried out by adding 2 µl Calf Intestinal Phosphatase (CIP) (Roche Diagnostics, Mannheim, Germany), 4 µl CIP buffer and 20 µl H₂O to a restriction digestion of 20 µl and incubating at 37°C for 1 h.

To construct new plasmids, linearized plasmid vectors and inserts with compatible overhanging ends or blunt-ends were ligated to form phosphodiester bonds. The reaction was carried out in 20 µl containing vector and insert in a molar ratio of 1:5, 2 µl T4 DNA ligase (MBI Fermentas, St. Leon-Roth, Germany), 2 µl 10×ligation buffer and 2 µl PEG 2000 (50% w/v in H₂O) and incubated at room temperature for 1-2 h.

2.7.4 Polymerase chain reaction

AmpliTaq Gold (Roche Diagnostics, Mannheim, Germany) was used to amplify and detect small amounts of specific DNA via PCR. This enzyme is a thermostable DNA polymerase, which was activated by incubation at 95°C for 10 min at the beginning of the PCR program. The reaction was run on thermocycler PCRSprint (Hybaid, MWG, Ebersberg, Germany). The reaction was carried out in a volume of 50 µl as follows: 5 µl 10×PCR buffer, 5 µl 2 mM dNTP, 5 µl primer 1 (10 µM), 5 µl primer 2 (10 µM), 5 µl plasmid DNA,

0,5 µl AmpliTaq Gold, H₂O ad 50 µl. To detect genomic DNA sequences, a bacterial colony was added via a pipette tip to the reaction mix instead of plasmid DNA.

PCR program (plasmid DNA):	1×10 min 95°C
	40× 20 s 94°C, 20 s 50°C, 30 s 72 °C
	1×10 min 72°C
PCR program (colony PCR)	1×10 min 95°C
	6× 20 s 94°C, 20 s 50°C, 30 s 72 °C
	35× 20 s 94°C, 20 s 56°C, 30 s 72 °C
	1×10 min 72°C

Subsequently, 5 µl of the PCR reaction were analyzed by gel electrophoresis. PCR products were purified using the “QIAquick PCR-Purification Kit” (Qiagen, Hilden, Germany) according to the manufacturer’s instructions

The “TOPO TA Cloning Kit” (Invitrogen, Groningen, The Netherlands) was used to clone PCR products into the pCR2.1-TOPO plasmid vector. The reaction was carried out according to the manufacturer’s instructions and the inserts were verified by sequencing (performed by GATC Biotech, Konstanz, Germany) using M13 Reverse and M13 Forward primer.

2.8 Microbiological methods

2.8.1 Long-term storage of bacteria

To store bacteria for a long-term period, 700 µl of a logarithmic growing culture were mixed with 300 µl glycerin (50% v/v in H₂O) and frozen at –70°C.

2.8.2 Transformation of chemically competent bacteria

For cloning procedures, aliquots of competent *E. coli* strains DH5 α and Top10 (Invitrogen, Groningen, The Netherlands) were thawed on ice, mixed with 10 μ l of a ligation reaction and incubated on ice for 10 min. Subsequently, the bacteria were transformed via the heat-shock method at 42°C for 45 s. 250 μ l SOC medium (provided by the manufacturer) were added immediately and the suspension was incubated at 37°C for 1-2 h. Finally, the bacterial suspension was plated on LB-agar plates containing selective antibiotics and incubated overnight at 37°C.

2.8.3 Transformation of electrocompetent bacteria

To prepare electrocompetent *E. coli* DH5 α and SM10 λ pir and *Salmonella* for the transformation with plasmid DNA, the strains were grown in LB medium at 37°C or 30°C (SM10 λ pir) and 180 rpm until an optical density (OD₆₀₀) of approximately 0,8 was reached. The bacteria were harvested by centrifugation at 4°C and 3000 \times g for 5 min and washed twice with ice-cold H₂O. Finally, the pellet was resuspended in 50 μ l ice-cold H₂O, mixed with 2 μ l plasmid DNA and transferred into a cuvette for electroporation. The electroporation was carried out using ice-cold 0,2 cm cuvettes (Biorad, Munich, Germany) and a “Gene Pulser II” (Biorad). Electroporation parameters were 2,5 kV, 25 μ F and 200 Ω (DH5 α) and 400 Ω (*Salmonella*) respectively. Subsequently, the bacteria were resuspended in 1 ml LB medium, shaken at 37°C or 30°C (SM10 λ pir) for 1 h before plating on selective LB-agar plates and incubation overnight at 37°C or 30°C (SM10 λ pir).

2.8.4 Preparation and transformation of chemically competent DH5 α λ pir

Chemically competent DH5 α λ pir were prepared according to the protocol of Inoue H. et al. (Inoue et al. 1990). Bacteria were incubated overnight on LB-agar plates at 28°C and 10-12 colonies were used to inoculate 400 ml SOB medium (Sambrook et al. 1989). The suspension was shaken in a 2 l Erlenmeyer flask at 18°C and 200 rpm until it reached an OD₆₀₀ of 0,6. After cooling on ice for 10 min, the bacteria were harvested by centrifugation at 4°C and 3000 \times g for 10 min. The pellet was resuspended in 80 ml ice-cold TB buffer (0,604 g Pipes; 0,442 g CaCl₂; 3,72 g KCl; 189 ml H₂O; pH adjusted to 6,7 with KOH; 11 ml 1 M MnCl₂) and 1 ml DMSO was added drop-wise before incubating again on ice

for 10 min. Aliquots (1 ml per sample) were stored at -70°C . For transformation, the aliquots were thawed on ice and 100 μl of the competent cells were mixed with 10 μl of a ligation reaction and incubated on ice for 30 min. Subsequently, the bacteria were incubated at 42°C for 45 s and afterwards on ice for 2-3 min. 1 ml LB medium were added and the suspension was incubated at 30°C for 1-2 h. Finally, the bacteria were plated on LB agar plates containing selective antibiotics and incubated overnight at 30°C .

2.8.5 Determination of reporter gene expression and plasmid amplification *in vitro*

The recombinant *Salmonella* strains were cultured in LB medium overnight, washed twice in M9, resuspended in IM and used to inoculate LB medium and IM at 1:100 dilution. The cultures were grown at 200 rpm for 6 h before being submitted to plasmid preparation, flow cytometry, luciferase assay or SDS-PAGE/Western Blot. Furthermore, the colony forming units (cfu) per ml of the culture were determined by plating serial dilutions. Plasmid DNA was extracted from 3 ml culture by mini plasmid preparation. The amount of DNA per ml culture was determined by comparison with marker DNA after digestion with appropriate restriction enzymes. The number of plasmid molecules per ml culture was calculated using the formula: 1 μg of 1000 bp DNA = $9,1 \times 10^{11}$ molecules. To determine the plasmid copy number per cfu, the number of plasmid molecules per ml was divided by the cfu per ml. To prepare samples for the luciferase assay, 500 μl of the cultures were washed once with PBS (Sambrook et al. 1989), resuspended in lysis buffer (25 mM Tris-HCl, 2 mM DTT, 10% glycerin, 1% Triton-X, 1 mg ml^{-1} lysozyme in H_2O) and incubated at room temperature with shaking for 10 min. Luciferase assays of the lysates were performed by using the Promega Luciferase Assay System (Promega, Mannheim, Germany) according to the manufacturer's specifications. In brief, 10 μl of sample were mixed with 100 μl of the Luciferase Assay Reagent (LAR) and the emission of light was subsequently measured during a 10 s time interval in a Lumat LB9507 luminometer (Berthold Technologies, Australia) as relative light units (RLU). To prepare samples for SDS-PAGE/Western Blot, bacterial pellets were lysed by sonification after adding 2 \times loading buffer (1,5 g Tris pH 6,8, 20 ml glycerine, 46 ml 10% w/v SDS, 10 ml β -mercaptoethanol, 4 ml 0,05% w/v bromphenolblue in 100 ml H_2O).

2.8.6 Tn7-mediated transposition in *Salmonella*

The chromosome of *E. coli* strain SM10 λ pir carries the transfer elements of the mobile plasmid RP4 (Simon et al. 1983) as well as the prophage λ pir (Miller and Mekalanos 1988). The carrier plasmids are derivatives of pSLE1f that are suitable for the site-specific insertion of foreign DNA into the genome of gram-negative bacteria. These plasmids contain the mobilizing region of plasmid RP4 and the foreign DNA between the transposon ends Tn7L and Tn7R. The helper plasmid pUX-BF13 provides the Tn7 transposition functions in trans as well as the mobilizing region of plasmid RP4 (Bao et al. 1991). Importantly, due to the λ pir-dependent R6K origin of replication, both plasmids can only be propagated in strains, which express *pir*, e.g. DH5 α λ pir and SM10 λ pir, not in the target strain SL7207. Tn7-mediated transposition in SL7207 was carried out according to the filter conjugation method (de Lorenzo et al. 1990). Briefly, the helper plasmid as well as the carrier plasmid was used for transformation of SM10 λ pir. Both strains and SL7207 were then grown at 28°C in LB medium until they reached OD₆₀₀ 0,8. Then 200 μ l of each culture were mixed, washed in LB medium, resuspended in 20 μ l LB medium and incubated on a 0,45 μ m cellulose filter (ME25, Schleicher&Schüll, Dassel, Germany) on a LB-agar plate at 28°C overnight. Streptomycin- and kanamycin-resistant clones that were sensitive to ampicillin, indicating loss of helper and carrier plasmid, were selected and tested by colony PCR for chromosomal integration using primer AEN32 and AEN43.

2.8.7 Determination of plasmid stability *in vitro*

Transformed *Salmonella* strains were cultured in LB medium at 37°C and 180 rpm overnight and passaged for 5 days every 12 h at 1:100 dilution in fresh medium with and without antibiotic selection. The cfu of the respective cultures were determined by plating serial dilutions. The stability was calculated by dividing cfu on antibiotic agar plates by total cfu on LB-agar without selecting antibiotics.

2.8.8 Flow cytometry of bacteria

2.8.8.1 Flow cytometry of bacterial cultures

To measure the *in vitro* expression of Gfp or Gfp_ova, 5 µl of bacterial cultures were analyzed in 1 ml buffer (2 mM EDTA in PBS) for scattering properties and green fluorescence without compensation using a FACSCalibur and WinMDI software.

2.8.8.2 Flow cytometry of bacteria from infected cells and mice

Bacteria from infected cells and mice were analyzed by two color flow cytometry (Bumann 2002). This method allows distinguishing GFP expressing bacteria from autofluorescent cellular debris since GFP expressing *Salmonella* have a substantially lower orange/green emission ratio (Fig. 2.9). Additionally, forward and sideward scatter were used to distinguish *Salmonella* from larger particles by setting an appropriate scatter gate.

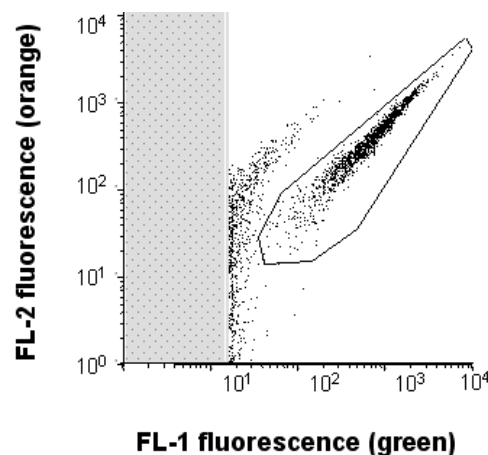


Fig. 2.9: Two color flow cytometry of *S. typhimurium* expressing GFP. A threshold value (grey shaded area) and a gate were set in the orange/green acquisition plot for detection of GFP expressing bacteria in the lysates of cells and organs.

2.8.8.3 Live/dead staining of bacterial cultures for flow cytometry

Bacterial live/dead staining was carried out using the BD Cell Viability Kit (BD Bioscience, Erembodegem, Belgium,) according to the manufacturer's instruction. This assay is based on staining the bacteria with two reagents: the membrane permeable dye

thiazole orange (TO) and the membrane impermeable dye propidium iodide (PI). Both dyes intercalate into the DNA thereby increasing fluorescence intensity.

2.9 Tissue culture

2.9.1 Determination of viable cells

An aliquot of a cell suspension was diluted 1:10 or 1:2 with 0,5% w/v trypanblue in PBS and 10 μ l of this mixture were analyzed in a Neubauer chamber. Trypanblue stains only dead cells with a leaky membrane; viable cells remain unstained and were counted. The number of cells ml^{-1} equals the number of viable cells within a big square multiplied with the dilution factor and 10^4 .

2.9.2 Detachment of adherent cells

Adherent cells were detached from culture dishes for counting and transfer. Cells were washed once with PBS and incubated at 37°C after adding trypsin-EDTA (TE) solution (0,5 g ml^{-1} trypsin; 0,2 g ml^{-1} EDTA in PBS) until the cells were detached. Subsequently, TE was inactivated by addition of IMDM containing FCS and the cells were separated by careful repeated pipetting.

2.9.3 Cryoconservation and thawing of cells

Cells from a dense growing 12- or 6-well plate were suspended in 0,5 ml ice-cold freezing medium (10% v/v DMSO in FCS), frozen at -70°C and stored in liquid nitrogen. Frozen cells were thawed at 37°C in a water bath, resuspended in prewarmed medium and washed twice before culturing.

2.9.4 CFSE labeling

To monitor *in vivo* cell proliferation or *in vivo* killing, cells were labeled with CFSE. After determining the cell number, cells were stained with the Vybrant CFDA SE (CFSE) cell tracer kit (Molecular Probes/Invitrogen, Karlsruhe, Germany) at 5×10^7 cells per ml CFSE in PBS for adoptive transfer experiments and *in vivo* cytotoxicity assays. The suspension was incubated at 37°C for 10 min, 25 ml complete IMDM were added and an incubation

step on ice for 5 min was performed. Subsequently, cells were centrifuged at 1000 rpm for 10 min and washed twice with PBS.

2.10 Biochemical methods

2.10.1 SDS-PAGE

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) separates proteins under denaturing conditions by molecular weight. In this study, 12% polyacrylamide gels were used. To prepare the resolving gel, 3,3 ml H₂O, 4 ml 30% acrylamide (Rotiphorese Gel 30, Roth, Karlsruhe, Germany), 2,5 ml 1,5 M Tris pH 8,8, 0,1 ml 10% w/v SDS, 0,1 ml 10% w/v APS and 0,004 ml TEMED were mixed. The stacking gel consisted of 2,7 ml H₂O, 0,67 ml 30% acrylamide, 0,5 ml 1 M Tris pH 6,8, 0,04 ml 10% v/v SDS, 0,04 ml 10% w/v APS and 0,004 ml TEMED. Before loading, the samples were denaturized at 95°C for 5 min. Rainbow-Marker RPN 756 (Amersham Biosciences, Freiburg, Germany) was used as protein marker for Western Blots. The gels were run at 150 V.

2.10.2 Western Blot

Western Blot is a method to specifically detect separated proteins on gels. After gel electrophoresis, the proteins were transferred on a 0,45 µm polyvinylidene fluoride transfer membrane (Immobilon-P, Millipore, Schwalbach, Germany) using the semi-dry method. The transfer membrane and Whatman 3 MM paper was soaked in blotting buffer (5,8 g Tris base, 28,5 g glycine, 1,0 g SDS, 200 ml methanol in 1 l H₂O) and the blot was assembled in the following order, starting from the bottom electrode plate (the cathode): 3 layers of wet Whatman paper, polyacrylamide gel, wet transfer membrane, 3 layers of wet Whatman paper. After removing air bubbles, the upper electrode was placed on top of the stack and the blot was run with 1 mA/(cm² of gel) for 1 h. Then the membrane was incubated in blocking buffer (5% w/v skim milk powder in 0,1% v/v Tween 20/PBS) at room temperature for 1-2 h before the first antibody (polyclonal rabbit-anti-LLO) was added in 1:3000 dilution in staining buffer (3% w/v skim milk powder in 0,1% v/v Tween 20/PBS) for 1 h at room temperature. After 3 washing steps with 0,1% v/v Tween 20/PBS, the proteins were stained with the secondary antibody (goat-anti-rabbit-horseradish peroxidase, 1:6000 dilution) at room temperature for 1 h. Subsequently, the

membrane was washed and specific bands were detected using the ECL-chemiluminescence kit (Amersham Biosciences, Freiburg, Germany) according to the manufacturer's instructions.

2.10.3 Determination of L-arabinose concentration in blood plasma

The concentration of L-arabinose in blood plasma of mice was enzymatically determined by the method of Melrose and Sturgeon (Melrose and Sturgeon 1983). In brief, 200 μl blood were obtained by retro orbital bleeding and 25 units heparin sodium were added immediately. Samples were centrifuged at 10000 x g for 5 min and 50 μl of the supernatant were mixed with 830 μl of 0,1 mM Tris-HCl (pH 8,6) and 100 μl of 5 mM nicotinamide-adenine dinucleotide (β -NAD, Sigma-Aldrich). Absorbance at 339 nm (A_1) was measured using the Spectronic 401 photometer (Milton Roy) and subsequently 20 μl 5 U ml^{-1} galactose dehydrogenase (Roche Diagnostics, Mannheim, Germany) were added. After incubating the reaction mixture at room temperature for 40 min, measurement (A_2) was repeated. The concentration of L-arabinose was calculated from the change of absorbance (A_2-A_1) multiplied with factor 475,9.

2.11 Immunological methods

2.11.1 Flow cytometry

Flow cytometry measures the properties of individual particles, e.g. the size and granularity of cells and bacteria. Moreover, by staining cells with appropriate fluorochrome-conjugated antibodies, this method allows sensitive detection of cell surface molecules. Cells for flow cytometric analysis were obtained from cell cultures or from homogenized organs. In general, single cell suspensions were prepared in FACS buffer (2% FCS, 2 mM EDTA in PBS) at a density of 10^6 – 10^7 cells ml^{-1} in 96-well plates or FACS tubes. To block Fc-receptors, cells were then treated with rat-anti-mouse CD16/CD32 (Fc γ III/II-receptor) (BD Pharmingen, San Diego, USA) on ice for 15 min. After washing, cells were stained with antibodies on ice for 15 min and washed once. Biotinylated antibodies were then counterstained with conjugated Streptavidin-APC-Cy7 (BD Pharmingen) on ice for 15 min. Cells were again washed once and resuspended in FACS buffer. Before analysis, 1 μg ml^{-1} propidium iodide (Sigma-Aldrich, Taufkirchen, Germany) was added to discriminate live and dead cells. For analysis a FACSCalibur or a

FACSCanto Flow Cytometer (Becton Dickinson, Heidelberg, Germany) were used and data were analysed using BD CellQuest Pro, BD FACSDiva or WinMDI software.

2.11.2 Antibodies for flow cytometry

rat-anti-mouse CD4 APC (RM4-5, BD Pharmingen)

anti-mouse DO11.10 TCR biotin (KJ1-26, Caltag Lab., Burlingame, CA, USA)

2.11.3 Magnetic cells sorting

Magnetic cell sorting (MACS) was developed to separate magnetically labeled cells from unlabeled cells by retaining them on a column in a magnetic field. In this study a CD4⁺ T Cell Isolation Kit together with LS Columns and a MidiMACS Separator (Miltenyi Biotec, Bergisch Gladbach, Germany) was used according to the manufacturer's instructions. Briefly, non-CD4⁺ T cells are labeled with a mix of biotin-conjugated antibodies and anti-biotin antibodies conjugated to magnetic beads. By applying the cell suspension onto the columns within a magnetic field, unlabeled CD4⁺ T cells pass through and can be isolated via negative selection. This method was used in the present work to obtain highly pure CD4⁺ T cells from DO11.10 mice for adoptive transfer experiments.

2.12 Determination of reporter gene expression in mouse macrophages

2.12.1 Infection of macrophages with recombinant Salmonella

Cells of the macrophage line J774-A.1 were seeded in 6-well plates at a density of 2×10^5 cells/well in IMDM medium without antibiotics one day before infection with recombinant Salmonella. Bacteria were grown overnight in LB medium at 37°C without shaking to an OD₆₀₀ of 0,8. After washing once with PBS, the bacteria were resuspended in antibiotic-free IMDM and the cells were infected with a multiplicity of infection (MOI) of 10. Cells were incubated at 37°C for 1 h, washed twice with PBS and extracellular bacteria were killed by incubation with medium containing 50 µg ml⁻¹ gentamycin (Biochrom, Berlin, Germany). The medium was replaced after 1 h with medium containing 10 µg ml⁻¹ gentamycin and incubation continued for 22 h to measure plasmid amplification by *in vivo* inducible promoters. To test L-arabinose inducible plasmid amplification, the sugar was

dissolved in medium and added at different concentrations into wells 3 h p.i. The cells were incubated for another 4 h before reporter gene expression was analyzed.

2.12.2 Preparation of infected mouse macrophages for flow cytometry

After washing twice with PBS, infected J774-A.1 cells were lysed in PBS containing 0,1% v/v Triton-X and incubated at 37°C for 10 min. Then, an equal volume of buffer (2mM EDTA in PBS) was added and the cell extract was filtered using 30 µm CellTrics (Partec GmbH, Münster, Germany) before samples were analyzed via two color flow cytometry.

2.12.3 Preparation of infected mouse macrophages for luciferase assay

After washing twice with PBS, infected J774-A.1 cells were detached in 1 ml PBS using a cell scraper. 500 µl of the sample were mixed with an equal volume of lysis buffer (50 mM Tris-HCl pH 8,3, 4 mM DTT, 20% v/v glycerol, 2% v/v Triton-X, 2 mg ml⁻¹ lysozyme) and the mixture was incubated at room temperature with shaking for 10 min. Luciferase activity of the supernatants was determined using the Luciferase Assay System according to the manufacturer's instructions. To determine intracellular bacterial numbers, 500 µl of detached cells were lysed by adding PBS containing 0,5% v/v Triton-X and cfu were enumerated by plating serial dilutions.

2.13 Immunization with recombinant Salmonella

2.13.1 Infection of mice

Recombinant Salmonella strains for i.v. infection were cultured in LB medium until they reached OD₆₀₀ 0,8 – 1,0. The bacteria were harvested by centrifugation at 6000 rpm for 5 min, washed twice in PBS and resuspended in PBS to $5 \times 10^{5-7}$ cfu ml⁻¹. A sample of 100 µl of this suspension was administered i.v. to mice. The bacteria for oral infection were grown in LB medium containing 1,5% NaCl until they reached OD₆₀₀ 0,8. After centrifugation, the bacterial suspension was adjusted to a concentration of $5-10 \times 10^9$ cfu ml⁻¹ in PBS and mice were inoculated with 100 µl intragastrically using a

gavage needle. Prior to immunization, 100 µl of 6,6% sodium bicarbonate in PBS was administered intragastrically immediately before.

2.13.2 Colonization and plasmid stability *in vivo*

At different time points after oral application of the different recombinant *Salmonella* strains, Peyer's Patches (PP), mesenteric lymph nodes (MLN) and spleen were removed and homogenized in PBS containing 0,1% Triton-X to lyse the cells and release intracellular bacteria using a Polytron PT3000 homogenizer (Kinematica AG, Littau-Lucerne, Switzerland). Serial dilutions were plated on LB-agar plates with or without antibiotics to determine the organ colonization and to calculate the percent of plasmid stability.

2.13.3 Preparation of infected mouse organs for flow cytometry

To determine reporter gene expression of bacteria *in vivo*, mice were sacrificed two days after i.v. infection. The spleen was removed and flushed with 2 ml PBS. Then, the suspensions were diluted 1:10 in 0,1% Triton-X/PBS containing 2 mM EDTA, filtered using 30 µm CellTrics and 300 µl of the samples were analyzed via two color flow cytometry.

2.13.4 Adoptive transfer of DO11.10 T cells and immunization with Ova₃₂₃₋₃₃₉ expressing Salmonella

DO11.10 mice were used as cell donors for adoptive transfer into sex-matched BALB/c mice. Lymphocytes were isolated from MLN and spleen, the CD4⁺ T cell population was isolated by MACS and labeled with 5 µM CFSE before transferring 5×10^6 transgenic CD4⁺ T cells per recipient mouse by tail vein injection. One day later, the mice were immunized orally or i.v. with 1×10^9 cfu or $0,5-1 \times 10^6$ cfu, respectively. As positive control, mice were fed 15 mg ovalbumin (Grade III, Sigma-Aldrich, Taufkirchen, Germany) in 200 µl PBS by gavage needle. Mice were sacrificed 7 d after immunization and single-cell suspensions of spleens and MLN were prepared by mincing through a 70 µm nylon mesh (BD Biosciences, Erembodegem, Belgium). Spleens were treated with erythrocyte lysis buffer (17 mM Tris, 140 mM NH₄Cl in H₂O, pH 7,2) for 5 min and taken up in complete IMDM containing DNase I (20 U ml⁻¹, Sigma-Aldrich, Taufkirchen,

Germany) whereas MLN were treated immediately with IMDM containing DNase I at 37°C for 15 min. After washing with FACS buffer and blocking, the cells were stained with biotinylated anti-tgTCR clonotype-antibody KJ1-26 and CD4-APC, followed by Streptavidin-APC-Cy7 and FACS analysis was performed on a FACSCanto Flow Cytometer.

2.13.5 *In vivo* cytotoxicity assay for LLO₉₁₋₉₉ specific immune responses

2.13.5.1 Immunization of mice

For *in vivo* cytotoxicity assays, mice were immunized with LLO₄₉₋₃₆₁ expressing Salmonella. Mice received three doses of recombinant Salmonella in 14 day intervals orally or i.v. with 1×10^9 cfu or $0,5-1 \times 10^5$ cfu, respectively. The assay was performed 9-11 days after the last immunization. Control mice were uninfected or infected with 1×10^3 *L. monocytogenes* i.v. 10 days before the assay. Practically, Listeria were incubated overnight at 180 rpm and 37°C in BHI broth. Next morning the culture was diluted 1:10 in fresh BHI broth and grown for approximately 2 h. Concentration of bacteria was determined by measuring the OD₆₀₀. Then the bacteria were washed in PBS and resuspended in PBS obtaining 1×10^3 ml⁻¹. Bacterial numbers were confirmed by plating serial dilutions of the suspension.

2.13.5.2 *In vivo* cytotoxicity assay

The *in vivo* cytotoxicity assay was performed to directly evaluate the induction of specific cytotoxic T cells in immunized mice. Naïve BALB/c mice (1 mouse per 3 recipient mice) were sacrificed and spleens were collected. The spleens were flushed with erythrocyte lysis buffer and erythrocytes were lysed at room temperature for 5-10 min. Cells were filtered through a 30 µm Nylon mesh (BD Biosciences, Erembodegem, Belgium), collected in 50 ml Falcon tubes and the tube was filled with complete IMDM before centrifuging at 1000 rpm for 10 min. Cells were resuspended in 50 ml complete IMDM and counted. The cell suspension was divided into two equal volumes. One half served as control cells and an equal volume of medium was added to the cells. The other half was sensitized with LLO₉₁₋₉₉ peptide at an end concentration of 1 µg ml⁻¹. The peptide was added to the cells in an equal volume of complete IMDM. Both control and sensitized cells were incubated at

37°C for 45 min. Cells were then washed twice with PBS. Subsequently, LLO-sensitized cells were stained with 5 μm CFSE at 5×10^7 cells per ml CFSE solution. Control cells were similarly stained but with 0,5 μm CFSE. Cells were incubated at 37°C for 10 min, 25 ml complete IMDM was added and a last incubation step for 5 min on ice was performed. Cells were again washed twice with PBS and both populations were pooled and adjusted to a concentration of $1\text{--}2 \times 10^8 \text{ ml}^{-1}$. Each immunized mouse was injected with 100 μl i.v.

16-18 h after injection mice were sacrificed and 200 μl heart blood was collected in a syringe containing 100 μl Heparin (Liquemin, Roche Pharma, Reinach, Switzerland, 1:100 diluted in PBS). The blood/heparin mix was diluted with 800 μl 5 mM EDTA in PBS and 2 ml erythrocyte lysis buffer and incubated at room temperature for 5 min. The suspension was centrifuged at 1000 rpm for 5 min and the pellet was again treated with 2 ml erythrocyte lysis buffer and incubated at room temperature for 5 min. Then, the cells were washed twice with 2 ml PBS and resuspended in 200 μl FACS buffer and finally analyzed by flow cytometry. The ratios of control cells (CFSE_{low}) and LLO₉₁₋₉₉-sensitized cells (CFSE_{high}) in uninfected mice should be 50:50 (in percent). When a cytotoxic reaction occurs, sensitized cells are killed and therefore the ratio will change. Specific cytotoxic killing can thus be determined.

2.14 Infection of tumor-bearing mice

To establish a subcutaneous tumor in BALB/c mice, CT26 or TS/A cells were harvested by trypsin treatment, washed once with PBS and resuspended in PBS to obtain a single cell suspension of $1\text{--}2 \times 10^7 \text{ ml}^{-1}$. 50 μl were injected s.c. into each mouse. Mice bearing tumors of 5 - 8 mm in diameter (approximately at day 10 after tumor transplantation) were intravenously injected with 5×10^5 cfu of bacteria suspended in PBS. At different time points post-infection, L-arabinose dissolved in PBS was administered i.p. to mice. At various time points after injection, mice were sacrificed and tumor, spleen and liver were transferred into 3 ml of ice-cold PBS. Tissues were disrupted using a Polytron PT3000 homogenizer. To analyze colonization, 500 μl homogenate was diluted with 500 μl PBS containing 0,25 % (v/v) Triton X-100 and serial dilutions were plated with or without antibiotics. To measure luciferase expression, 500 μl homogenate were mixed with an equal volume of lysis buffer (50 mM Tris-HCl pH 8,3, 4 mM DTT, 20% v/v glycerol,

2% v/v Triton-X, 2 mg ml⁻¹ lysozyme) and the mixture was incubated at room temperature with shaking for 10 min. Luciferase activity of the supernatants was determined using the Luciferase Assay System according to the manufacturer's instructions.

2.15 Non-invasive *in vivo* imaging

BALB/c mice were immunized intragastrically with 2×10^9 cfu of SL7207 harboring the *luxCDABE* expression plasmid pAEN102. Mice were anaesthetized with isoflurane using the XGI-8 gas anaesthesia system (Xenogen, Hopkinton, USA). Images were obtained at consecutive time points thereafter using the IVIS-200 system (Xenogen) according to instructions of the manufacturer. The software Living image 2.6 (Xenogen) was used for image analysis and quantification of emission intensities.

3 Results

Thus far, optimization of *Salmonella* as delivery vector for proteins or expression plasmids with regard to stability of the recombinant phenotype included chromosomal integration of the gene of interest or the use of low-copy number plasmids in conjunction with balanced lethal systems (Kotton and Hohmann 2004). In the present work, as an alternative, a system for inducible amplification of expression plasmids was developed in the *S. typhimurium* vaccine strain SL7207. To allow control of the copy number, expression plasmids were constructed that contain two origins of replication. The first replicon ensures the minimal-copy state under non-inducing conditions (e.g. the inoculum). The second, medium-copy replicon is derived from the broad host-range plasmid RK2. It is responsible for conditional amplification of the expression plasmid (Wild et al. 2002). Replication via the RK2-derived origin of replication *oriV* depends on the protein TrfA, which is essential for replication initiation. TrfA binds in the monomeric form to the host initiator protein DnaA and to direct repeats (iterons) at *oriV* (Kongsuwan et al. 2006). Expression of the *trfA* gene is tightly controlled by a transcriptional linkage to *Salmonella*-specific *in vivo* inducible promoters or the L-arabinose inducible promoter P_{BAD} , respectively. Upon induction, the promoters drive the expression of the replication protein TrfA that in turn activates the second replicon and thereby mediates amplified plasmid replication.

3.1 Establishment of an *in vivo* inducible plasmid amplification system

3.1.1 Characterization of *in vivo* inducible promoters in *S. typhimurium* SL7207

First, *in vivo* inducible promoters were used to initiate plasmid amplification upon vaccine administration. As a prerequisite for tight control, suitable promoters should provide low background activity under non-inducing conditions coupled with a strong expression once being activated. Therefore, three previously identified *in vivo* inducible promoters P_{phoN} , $P_{stm1630}$ and P_{sifA} (Rollenhagen et al. 2004) were considered. First, they were characterized in SL7207, an attenuated strain of *S. typhimurium* that is commonly used as carrier for heterologous antigens and expression plasmids (Hoiseth and Stocker 1981). Promoter P_{phoN} drives the PhoP-activated expression of *phoN*, a periplasmic non-specific acid phosphatase

(Kasahara et al. 1991). Promoter P_{sifA} regulates the expression of the SPI2-TTSS effector SifA (Brown et al. 2006) and promoter $P_{stm1630}$ drives the expression of a putative inner membrane protein within SPI2. These promoters were chosen because they share the common feature to be activated within intracellular compartments of host cells. Thus, they have a high potential to mediate the amplification of expression plasmids upon entry of *Salmonella* into APC of lymphoid tissues.

Fragments containing the promoter regions of *phoN*, *stm1630* and *sifA* were cloned upstream of *gfpmut2* and integrated as a single copy into the Tn7-specific integration site of the *Salmonella* chromosome (see Fig. 2.8). This GFP variant is stable and accumulates in cells rendering the detection of low promoter activity feasible. The intracellular conditions leading to activation of the promoters *in vivo* can be mimicked *in vitro* by growing the bacteria in defined minimal media containing low amounts of divalent cations (Deiwick et al. 1999). Accordingly, to analyze reporter expression, the chromosomal integration derivatives were grown in induction medium, an acidic minimal medium without Ca^{2+} and low Mg^{2+} levels. Under inducing conditions, all strains bearing chromosomally integrated constructs expressed GFP in detectable amounts (Fig. 3.1). P_{sifA} showed the strongest induction (median fluorescence intensity MFI 5,5) in comparison to P_{phoN} (MFI 4) and $P_{stm1630}$ (MFI 3,9). Importantly, for P_{sifA} , almost no background was detectable when grown in LB medium compared to the parental strain SL7207. In contrast, slight activation of P_{phoN} and $P_{stm1630}$ during growth in LB medium could be observed.

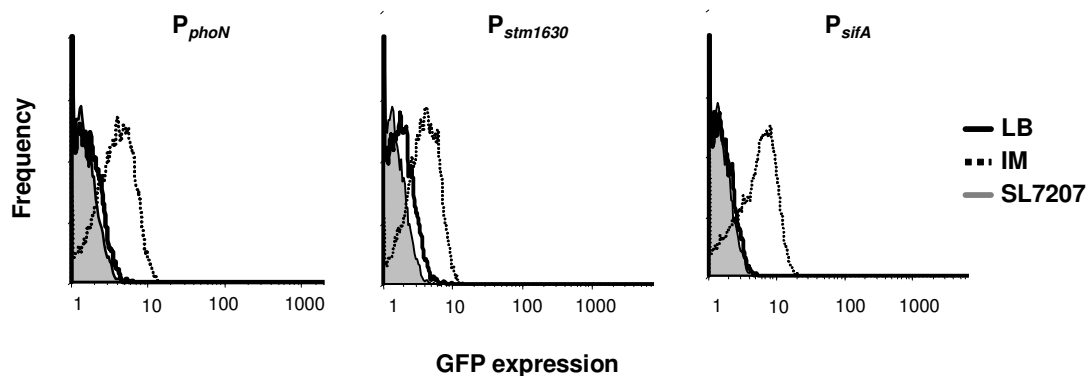


Fig. 3.1: Flow cytometric detection of GFP expression *in vitro*. Thick lines represent strains SL7207::AEN45.1 (P_{phoN} -gfp), SL7207::AEN45.2 ($P_{stm1630}$ -gfp) and SL7207::45.3 (P_{sifA} -gfp) grown under non-inducing conditions (LB), dashed lines represent strains grown under inducing conditions (IM). The shaded curve represents background fluorescence of the parental strain SL7207.

3.1.2 Construction of strains with inducible plasmid amplification

Next, the promoters were fused to *trfA* in order to amplify expression plasmids. *TrfA* cassettes were chromosomally integrated via Tn7-mediated transposition. Strains SL7207::AEN47.1-3 are shown in Fig. 2.8. Plasmid pAEN35 was cloned as reporter construct for plasmid amplification. This plasmid is equipped with the F plasmid derived single-copy maintenance system *oriS-repE-parABC* as well as the second origin of replication *oriV* (Fig. 3.2). Moreover, pAEN35 harbors *gfp_ova* under control of the β -lactamase promoter of *E. coli* P_{bla} . This weak promoter was chosen to obtain a sensitive method for detection of plasmid amplification via increased reporter expression. Furthermore, the instable GFP_ova variant was chosen as reporter in order to prevent excessive GFP accumulation thus impairing *Salmonella* virulence (Wendland and Bumann 2002).

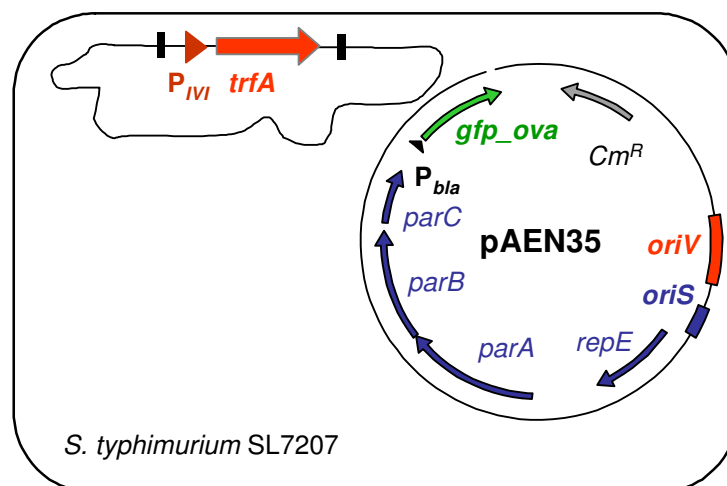


Fig. 3.2: Construction of SL7207 strains with inducible plasmid amplification. The bacteria carry a *gfp_ova* expression plasmid, which is maintained as single copy due to F plasmid elements. Upon promoter activation, the chromosomally integrated replication protein *TrfA* is expressed and mediates amplified replication from the second replicon, *oriV*.

Strains SL7207::AEN 47.1-3 were transformed with pAEN35, which is maintained as single copy when the promoters are not active. Upon induction of the *oriV/trfA* amplification system plasmid amplification should start. As expected, the copy number of plasmid pAEN35 increased in all strains when grown under inducing conditions compared to growth in standard medium (Fig. 3.3). The strain P_{sifA} -*trfA* induced the strongest

amplification with a low background under non-inducing conditions (from one to fifty plasmid copies).

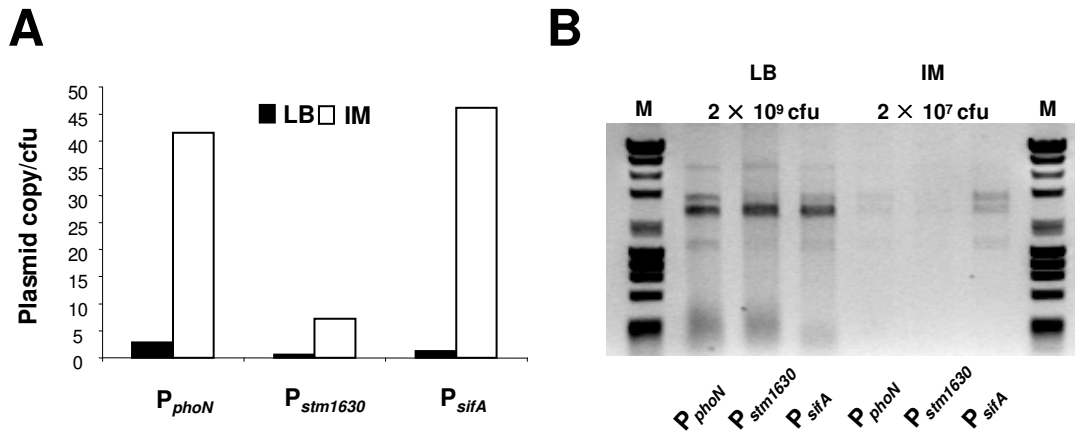


Fig. 3.3: *In vitro* inducible plasmid amplification. (A) Copy number of pAEN35 in strains SL7207::AEN47.1 (P_{phoN} -*trfA*), SL7207::AEN47.2 ($P_{stm1630}$ -*trfA*) and SL7207::47.3 (P_{sifA} -*trfA*) grown under non-inducing conditions (LB, black bars) and inducing conditions (IM, open bars). (B) Plasmid preparations from the different strains. A total of 2×10^9 transformants were used for plasmid preparations from strains grown in LB and 2×10^7 for IM, respectively. Plasmids were digested with *Pst*I to facilitate comparison on the agarose gel. M indicates the size marker.

The strains were further analyzed in culture for reporter gene expression via flow cytometry. Amplification of pAEN35 in P_{sifA} -*trfA* resulted in an increase of *gfp_ova* expression whereas almost no fluorescence could be detected in standard growth medium. This is also obvious when compared to the parental strain transformed with the same plasmid (Fig. 3.4 A). In contrast, numbers of expression plasmid and reporter expression were higher in strain P_{phoN} -*trfA* under non-inducing conditions compared to strain P_{sifA} -*trfA*, demonstrating the higher background activity of this promoter that could be already observed (see Fig. 3.1). Compared to P_{phoN} and P_{sifA} , the plasmid amplification induced by $P_{stm1630}$ was significantly lower.

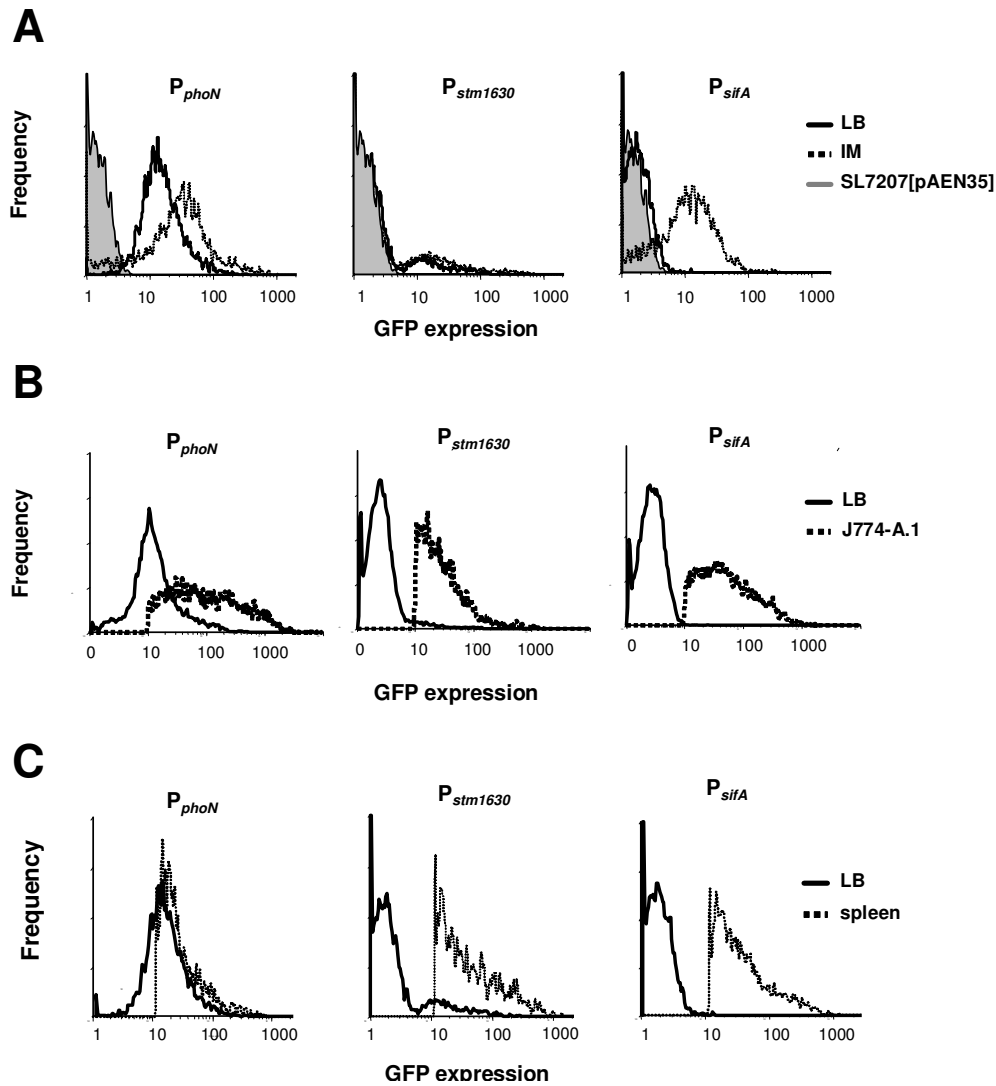


Fig. 3.4: *In vitro* and *in vivo* inducible plasmid amplification. Strains SL7207::AEN47.1 (P_{phoN} -trfA), SL7207::AEN47.2 ($P_{stm1630}$ -trfA) and SL7207::47.3 (P_{sifA} -trfA) harboring pAEN35 were used. (A) Flow cytometric detection of *in vitro* GFP expression. The shaded curve represents background fluorescence of the parental strain SL7207 transformed with pAEN35. (B) GFP expression of strains 24 h after infection of J774-A.1 mouse macrophages quantified by two color flow cytometry. (C) *In vivo* GFP expression of strains measured by two color flow cytometry. Mice were infected i.v. with 5×10^6 cfu; 2 d p.i. spleens were removed and analyzed. In each case, thick lines represent strains grown under non-inducing conditions (LB), dashed lines represent strains grown under inducing conditions (IM, macrophages, spleen).

The quantitation of GFP in infected host cells is normally hampered by strongly autofluorescing tissue fragments, which significantly decrease the sensitivity of detection. Therefore, two color flow cytometry was used to quantify the GFP expression of *Salmonella* after infection of the macrophage cell line J774-A1 and in spleens of infected mice (Bumann 2002). This method allows spectral distinction between GFP fluorescence

and background autofluorescence, resulting in lower detection thresholds for Salmonella GFP expression in infected tissues.

First, J774-A1 mouse macrophages were infected with strains SL7207::AEN47.1-3 harboring pAEN35, lysed 24 h thereafter and cellular lysates were analyzed by two color flow cytometry. As shown in Fig. 3.4 B, all three chromosomal constructs induced levels of GFP expression that were above the background fluorescence of cellular debris indicating a successful amplification of the expression plasmids.

Next, amplification of GFP_ova expression plasmids was analyzed *in vivo*. Mice were infected intravenously with the recombinant strains and two days later spleen homogenates were submitted to two color flow cytometry. Again all three strains induced plasmid amplification leading to enhanced levels of the reporter compared to strains grown under noninducing conditions (Fig. 3.4 C). Moreover, no bacteria expressing GFP above the background fluorescence of cellular debris could be detected in mice that were infected with parental SL7207 carrying the expression plasmid (data not shown). As can be seen in Fig. 3.4 B and Fig. 3.4 C, $P_{stm1630-trfA}$ exhibits similar levels of induced GFP expression as P_{sifA} and P_{phoN} , which is in contradiction to the data obtained in induction medium. Probably, bacterial subpopulations with lower GFP expression levels were not detected due to interfering host cell autofluorescence in this case.

Taken together, the data clearly show that expression plasmids can be amplified in bacterial culture, in J774-A1 mouse macrophages and *in vivo* using the novel system. However, it appears that P_{sifA} is the most appropriate promoter for the development of a vaccine strain, since it combines a strong inducible amplification in infected tissues with a low background in the uninduced state.

3.1.3 Simultaneous amplification of plasmid replication and antigen expression

In order to further increase antigen expression, a system for simultaneous amplification of plasmid replication and protein expression was established. Therefore, the inducible promoter P_{sifA} was used in parallel for the amplification of expression plasmids and in addition directly for antigen expression (Fig. 3.5). In doing so, enhanced levels of antigen

expression were expected. Additionally, the background fluorescence under non-inducing conditions should be further decreased using P_{sifA} -*gfp_ova* instead of P_{bla} -*gfp_ova*.

The use of low-copy expression vectors derived from plasmids pSC101, pMB1 or p15A had been described for *Salmonella* vaccines (Panthel et al. 2005, Bauer et al. 2005, Kotton et al. 2006). Such plasmids are maintained at 5-20 copies per cell imposing a low metabolic burden on transformed bacteria. To increase the starting copy number for amplification from 1 to 5-20, plasmids were generated harboring these different low-copy number replicons (*ori X* in Fig. 3.5).

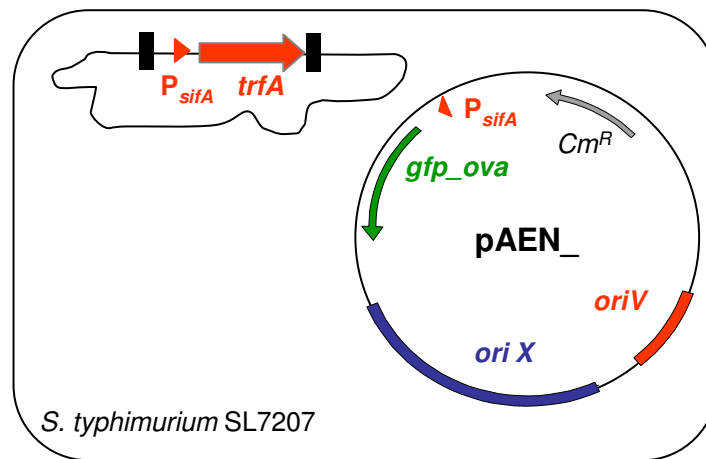


Fig. 3.5: Strategy for simultaneous amplification of plasmid replication and antigen expression using the *in vivo* inducible promoter P_{sifA} . First, once activated, the promoter drives the expression of *gfp_ova*. The reporter is encoded on expression plasmids that are maintained at different minimal-copy numbers depending on the *ori X*. Second, once activated, the promoter induces expression of TrfA, which mediates amplified replication from the second replicon, *oriV*.

The parental strain SL7207 and the chromosomal integration derivative P_{sifA} -*trfA* were transformed with these new plasmids and tested for antigen expression *in vitro*. Detectable antigen expression was induced by P_{sifA} in the parental strain SL7207 harboring the plasmid that is maintained as single copy due to the F plasmid replicon (Fig. 3.6 A). Enhanced antigen expression could be observed in induction medium with the strain SL7207:: P_{sifA} -*trfA*. This is due to amplification of the expression plasmid in addition to induced expression. Importantly, this expression plasmid was amplified up to 50 copies *in*

vitro like pAEN35, indicating that inducible expression of the antigen does not interfere with inducible plasmid amplification.

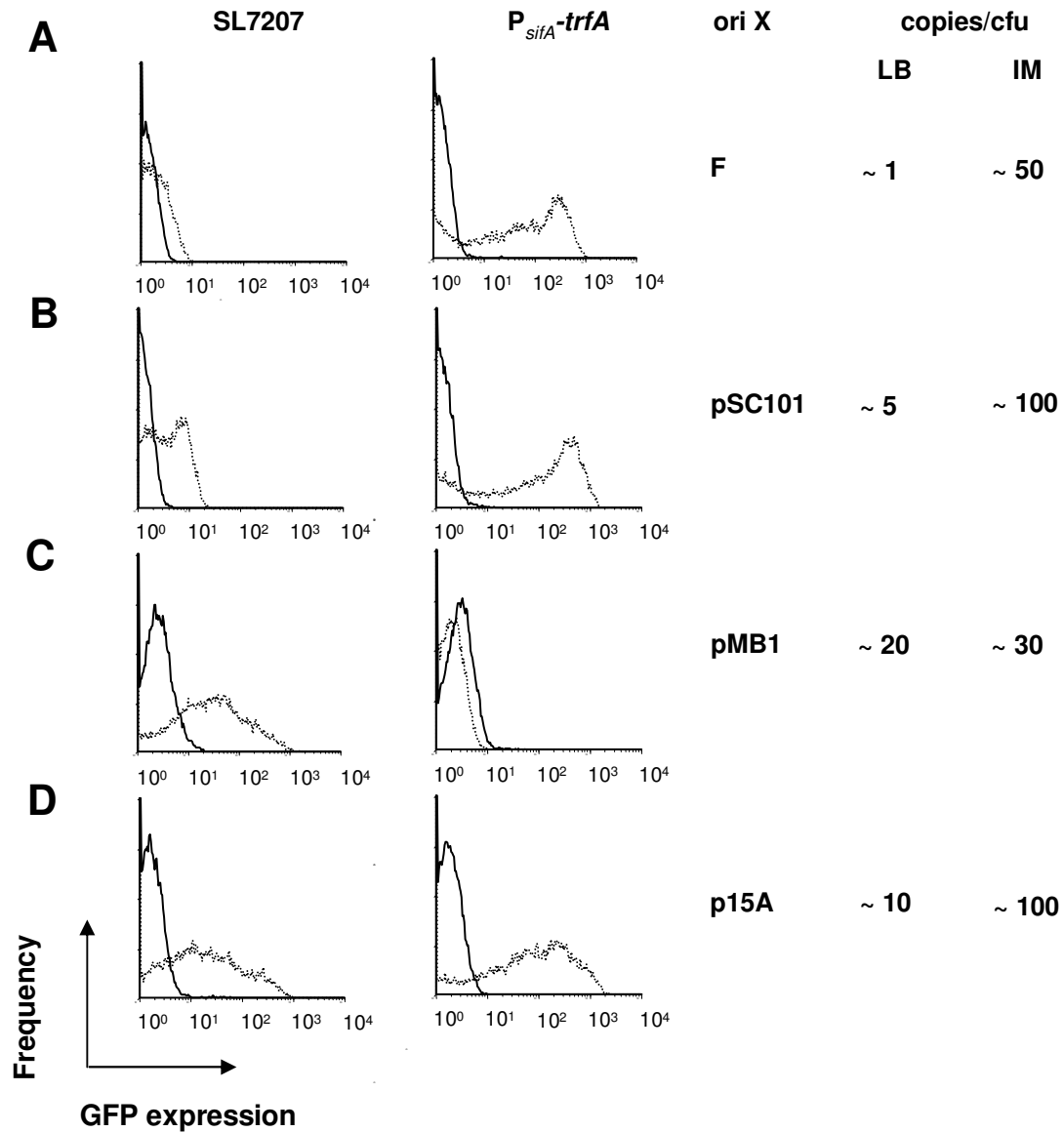


Fig. 3.6: Simultaneous amplification of plasmid replication and GFP_{ova} expression *in vitro* under control of P_{sifA} . Strains SL7207 or SL7207:: P_{sifA} -trfA harboring expression plasmids pAEN51 (A), pAEN61 (B), pAEN108 (C) or pAEN109 (D) were grown under non-inducing conditions (LB, thick lines) or inducing conditions (IM, dashed lines), analyzed by flow cytometry and the copy number was determined.

Exchanging the F plasmid replicon with other low-copy number replicons resulted in all cases in higher antigen expression in the parental strain SL7207 under inducing conditions (Fig. 3.6). Thereby, GFP_{ova} expression increased proportionally with the copy number of

the plasmids as expected. However, when strain SL7207::P_{sifA}-trfA was transformed with the indicated expression plasmids, different effects could be observed. In case of the pSC101 and p15A ori, a copy number of about 100 molecules in each bacterial cell could be determined in induction medium. This was associated with high GFP expression (Fig. 3.6 B and D). In contrast, the plasmid harboring the pMB1 ori did not show enhanced antigen expression and significant increase of copy number in SL7207::P_{sifA}-trfA under inducing conditions (Fig. 3.6 C). It is unclear why the pMB1 containing plasmid could not be amplified. Obviously, some interference of replication from the different origin of replications pMB1 and *oriV* takes place.

Taken together, an increased conditional antigen level could be achieved on the one hand by placing plasmid amplification and protein expression under simultaneous control of the *in vivo* inducible promoter P_{sifA}. A higher copy number could be achieved in some cases under inducing conditions by exchanging the origin of replication that determines the initial minimal copy number. This result was rather unexpected, since the second origin of replication *oriV* and the replication protein TrfA mediate the plasmid amplification. Therefore, its magnitude should be independent of the basal uninduced copy number.

The constructs pAEN51 and pAEN61, containing the F plasmid and pSC101 replicon, were chosen for further experiments, since they combine a very low copy number (single copy and 5 copies, respectively) and low antigen expression under non-inducing conditions with strong inducible plasmid amplification and antigen expression *in vitro*.

Hence, SL7207::P_{sifA}-trfA carrying pAEN51 and pAEN61 were used to infect mice intravenously and two days later spleen homogenates were analyzed for GFP expression via two color flow cytometry. Both constructs exhibited a strong antigen expression in spleens of infected mice (Fig. 3.7). The higher copy number of induced pAEN61 could be confirmed, since the GFP expression level of bacteria carrying this construct was higher (MFI 542) compared to plasmid pAEN51 (MFI 457).

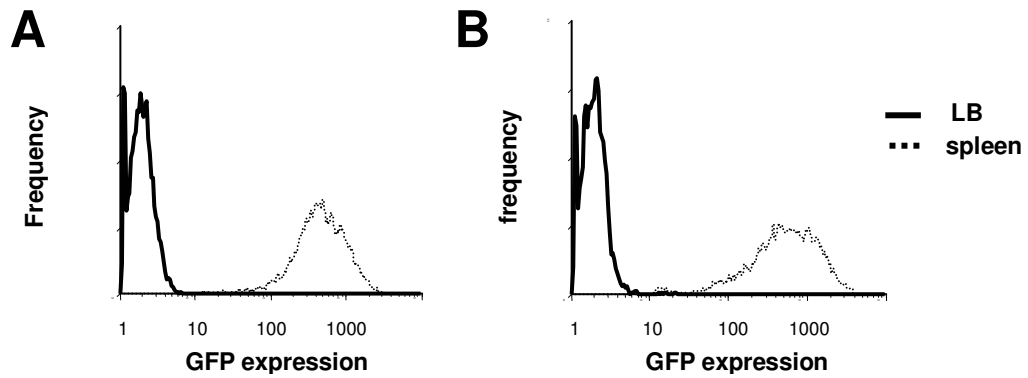


Fig. 3.7: Simultaneous amplification of plasmid replication and antigen expression *in vivo* under control of P_{sifA} . GFP expression of strain SL7207::AEN47.3 (SL7207:: P_{sifA} -*trfA*) harboring plasmid pAEN51 (A) and plasmid pAEN61 (B) measured by two color flow cytometry. Mice were infected i.v. with 5×10^6 cfu of the indicated strains. 2 d p.i. spleens were removed and analyzed. Thick lines represent bacteria grown under non-inducing conditions (LB); dashed lines represent bacteria grown *in vivo*.

3.1.4 Colonization of mice by strains with inducible plasmid amplification

To test whether the chromosomal integration of the *trfA* gene influences the invasion efficiency, mice were infected orally with the parental strain SL7207 or the plasmidless recombinant strain SL7207::AEN47.3 harboring a P_{sifA} -*trfA* cassette integrated into the chromosome. Similar bacterial numbers could be isolated from PP and mesenteric lymph nodes (MLN) at different time points after infection with both strains (Fig. 3.8).

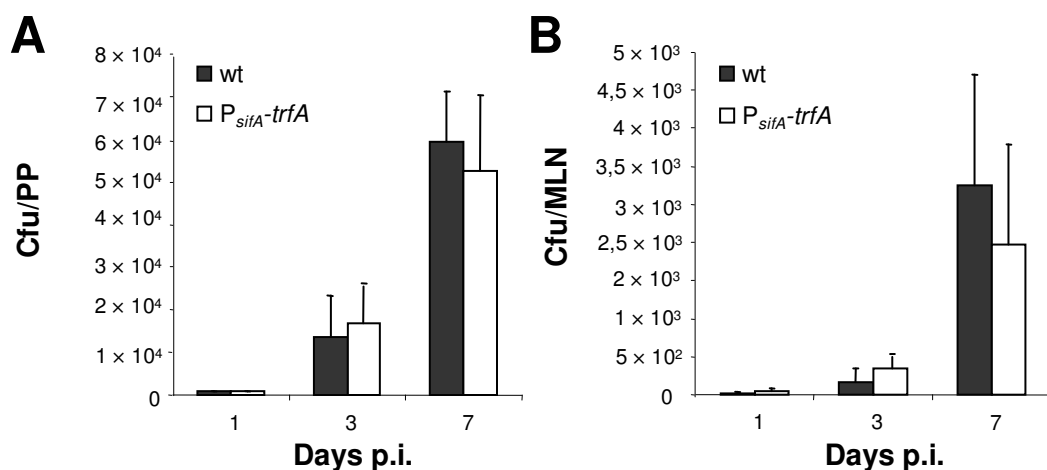


Fig. 3.8: Comparison of colonization efficiency of the parental strain SL7207 and the chromosomal integration derivative SL7207:: P_{sifA} -*trfA*. Bacterial numbers in PP (A) and MLN (B) after oral administration of 1×10^9 cfu of SL7207 or SL7207:: P_{sifA} -*trfA*. Values represent the average for three mice per group (mean + s.d.).

Besides the *in vivo* antigen expression level, colonization capability and stability of the vector system are important parameters for vaccination efficiency. To evaluate the $P_{sifA-trfA}$ strains with inducible plasmid amplification in this respect, mice were infected orally and then bacterial numbers and plasmid stability were determined in PP, MLN and spleen at different time points. Additionally, these strains were compared with the parental strain SL7207 without plasmid or transformed with the same $P_{sifA-gfp_ova}$ expression plasmids.

As seen in Fig. 3.9 A, the parental strain SL7207 exponentially proliferates in the PP until day 7 p.i.. A similar kinetic with about 10-fold lower numbers could be observed in MLN (Fig. 3.9 B). Bacterial numbers in spleen were similar to those observed in MLN at day 7 p.i. (data not shown). Despite lower bacterial counts at day 1 p.i., *Salmonella* without chromosomal integration carrying only the expression plasmids had no major colonization disadvantages in PP and MLN compared to SL7207. This confirmed that expression plasmids with the F plasmid and pSC101 replicon impose no metabolic burden upon the carrier when maintained in one or five copies, respectively. In concordance, the plasmids were present throughout the entire observation period (Fig. 3.10 A and B). The $P_{sifA-trfA}$ strains that amplified the expression plasmids exhibited comparable bacterial numbers to plasmid carriers without $P_{sifA-trfA}$ integration at day 1 p.i.. However, their numbers declined throughout the infection (Fig. 3.9 A and B). This could be attributed to plasmid loss within 7 days p.i. (Fig. 3.10. A and B), since the values in Fig. 3.9 A and B were determined by plating with antibiotic selection.

That the induced amplification would eventually lead to plasmid loss was not unexpected. Again not surprisingly, the pSC101 plasmid, which is amplified twice as high was lost more rapidly than the one with the F plasmid replicon.

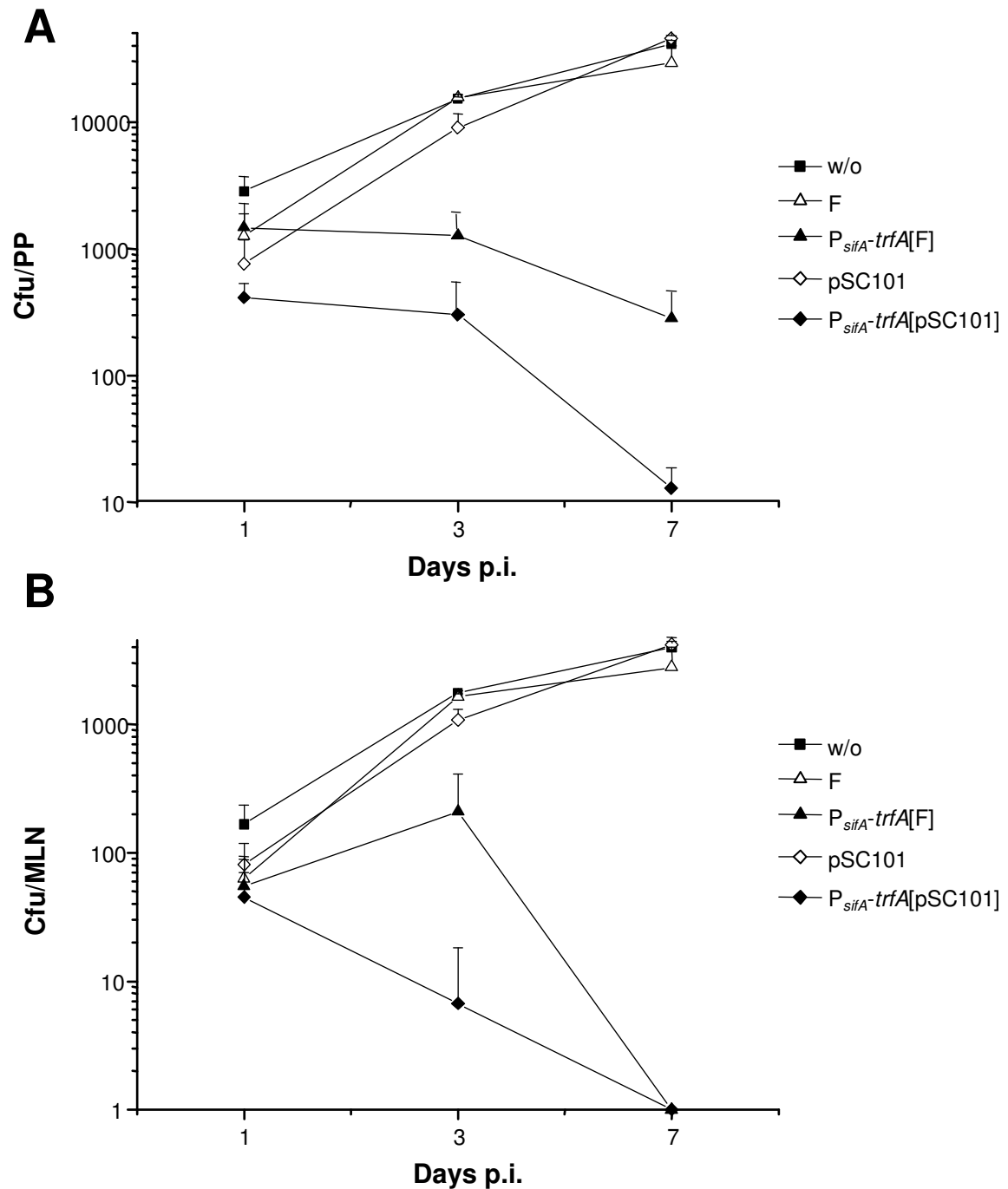


Fig. 3.9: Bacterial colonization after oral infection of mice. Mice were infected with 5×10^8 cfu of SL7207 or recombinant GFP_ova expressing SL7207. Colonization of PP (A) and MLN (B) as determined from plating with antibiotics. Values represent the average for three mice per data point (mean + s.d.). For clarity of the figure, plasmid constructs are indicated with the first origin of replication only: F = pAEN51; pSC101 = pAEN61. Strains with inducible amplification of the expression plasmid are indicated as P_{sifA} -trfA. Experiment was performed twice with similar results.

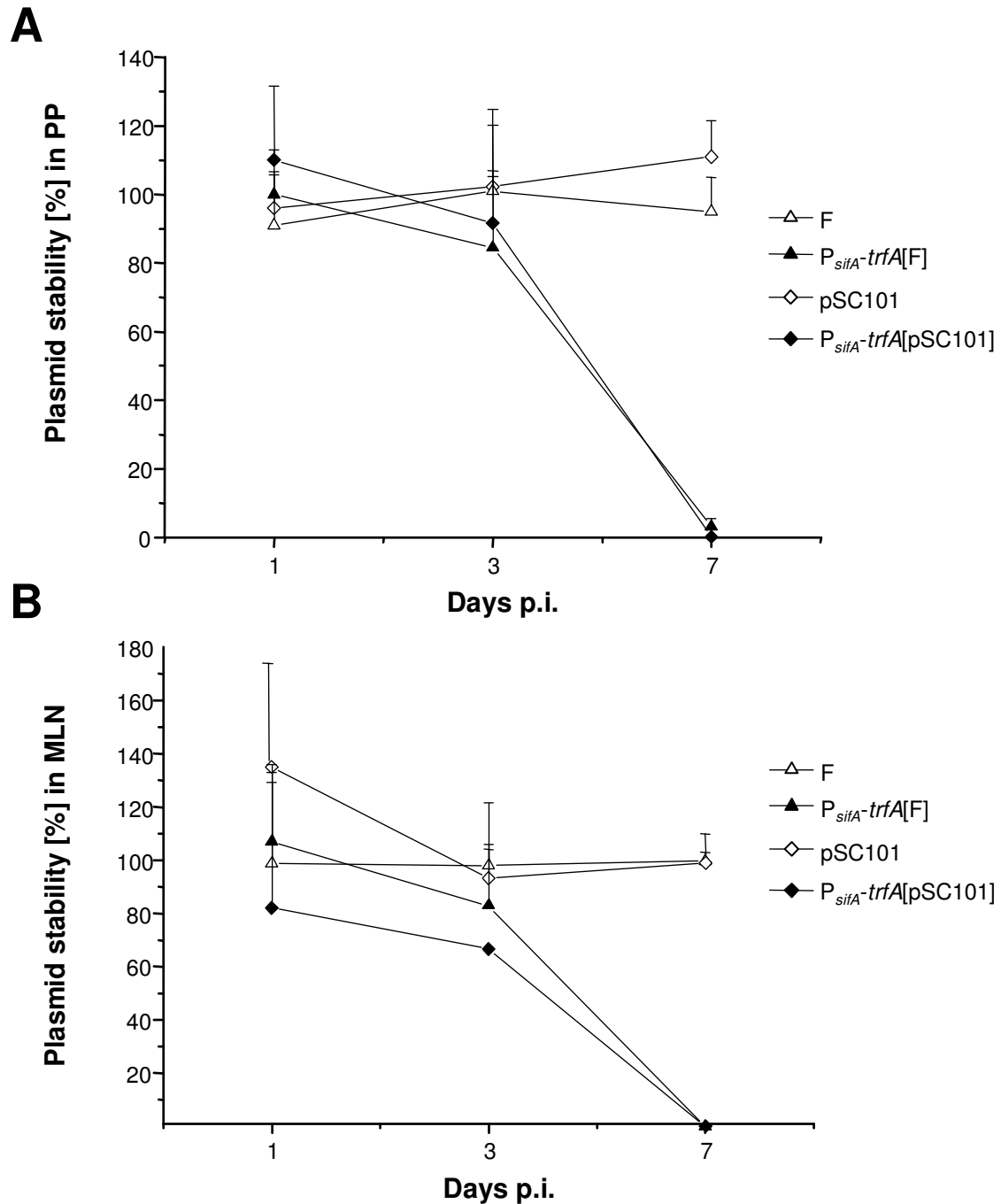


Fig. 3.10: Plasmid stability after oral infection of mice. Mice were infected with 5×10^8 cfu of recombinant GFP_{ova} expressing SL7207. Stability of P_{sifA} -gfp_{ova} expression plasmids in PP (A) and MLN (B). Values represent the average for three mice per data point (mean + s.d.). For clarity of the figure, plasmid constructs are indicated with the first origin of replication only: F = pAEN51; pSC101 = pAEN61. Strains with inducible amplification of the expression plasmid are indicated as P_{sifA} -trfA. Experiment was performed twice with similar results.

3.1.5 Immunization of mice

The colonization studies showed that the novel *Salmonella* strains with inducible plasmid amplification stably retained expression plasmids in a multi-copy state for at least three days *in vivo*. This represents an important improvement in comparison to high-copy number systems in which no *Salmonella* carrying expression plasmids could be detected in murine tissues after oral infection due to a very low stability (Darji et al. 1997, Dunstan et al. 2003, Bauer et al. 2005). Hence, it should be tested, whether this results in sufficient expression of the model antigens OVA₃₂₃₋₃₃₉ and LLO to trigger specific CD4⁺ and CD8⁺ T cells.

3.1.5.1 Generation of CD4⁺ T cell responses

GFP_ova had been used as reporter to measure *in vivo* inducible plasmid amplification coupled with antigen expression via flow cytometry. This gene is a fusion of the *gfp* variant *mut2* and a 25-amino-acid-fragment of ovalbumin. It contains a dominant MHC class II (H-2^d) presented 17-mer T cell epitope together with the 4 adjacent amino acids on both the N and the C termini (Bumann 2001a). Therefore, GFP_ova is a suitable antigen to analyze the induction of Ova-specific CD4⁺ T cells following immunisation with recombinant *Salmonella*. A widely used transgenic T cell receptor (tgTCR) adoptive transfer model (Pape et al. 1997) was used for analysis. In this system, a few million CD4⁺ T cells from DO11.10 mice, which are transgenic for a TCR that recognizes the ovalbumin epitope (amino acids 323 to 339) bound to I-A^d MHC class II molecules, are transferred into syngeneic BALB/c mice. In such chimeric mice, the small fraction of transgenic T cells can be traced with the clonotypic monoclonal antibody KJ1-26. Transgenic T cells were additionally labeled with the fluorescent dye CFSE prior to the adoptive transfer in order to characterize their *in vivo* division via flow cytometry. Proliferation of cells stained with CFSE can be followed as the dye is diluted upon cell division and the fluorescence intensity accordingly decreases.

In a pilot experiment, chimeric mice were immunized intragastrically and intravenously with SL7207::P_{sifA}-*trfA* harboring P_{sifA}-*gfp_ova* expression plasmid pAEN51 with the F plasmid replicon one day after the adoptive transfer. Control mice received SL7207 without any expression plasmid or ovalbumin orally. After oral administration of GFP_ova expressing SL7207 to chimeric mice, 12% of the ovalbumin-specific T cells in the spleen

lost part of their CFSE fluorescence (Fig. 3.11 C), indicating *in vivo* division. In contrast, only 5% dividing transgenic T cells could be observed after oral immunization with the negative control SL7207 (Fig. 3.11 B). Most of the cells had divided in the positive control using orally administered ovalbumin (Fig. 3.11 A). After i.v. administration of a lower dose of GFP_ova expressing SL7207 to chimeric mice, more dividing antigen-specific T cells could be detected in comparison to oral administration (Fig. 3.11 D). This can be attributed to a stronger colonization of the spleen after intravenous administration and was also observed in the MLN (data not shown). Since the *in vivo* division of transgenic T cells was stronger in case of intravenous administration of recombinant Salmonella, this route of immunization was chosen for further experiments to assess the effect of plasmid amplification on the generation of CD4⁺ T cell responses.

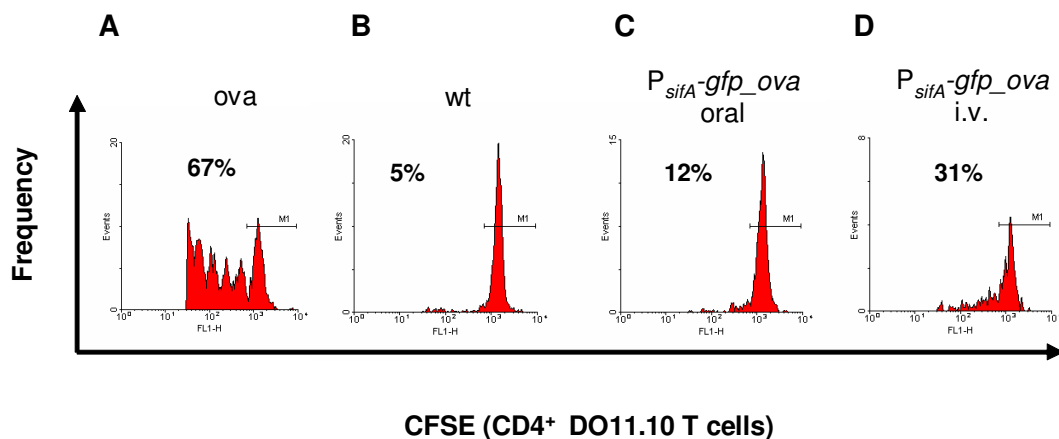


Fig. 3.11: Proliferation of Ova-specific T cells. Fluorescence of CFSE-labeled Ova₃₂₃₋₃₃₉-specific T cells 7 days after oral immunization with 15 mg ovalbumin (A), 1×10^9 cfu of SL7207 (B), and 1×10^9 cfu of SL7207::P_{sifA}-trfA[F:P_{sifA}-gfp_ova] (C) or after i.v. immunization with 5×10^5 cfu of SL7207::P_{sifA}-trfA[F:P_{sifA}-gfp_ova] (D). Spleen cells of two mice per group were pooled and histograms are gated on CD4⁺ and tgTCR positive lymphocytes. Experiment was performed once.

Next, chimeric mice were immunized intravenously with 5×10^5 cfu SL7207::P_{sifA}-trfA harboring P_{sifA}-gfp_ova expression plasmids pAEN51 (F plasmid replicon) and pAEN61 (pSC101 replicon) one day after the adoptive transfer. These strains were compared with the parental strain SL7207 and SL7207 transformed with the same P_{sifA}-gfp_ova expression plasmids. As positive control, 15 mg ovalbumin were administered orally. Spleen cells as well as lymphocytes from mesenteric lymph nodes were tested for proliferation of antigen-specific helper T cells via flow cytometry 7 d p.i..

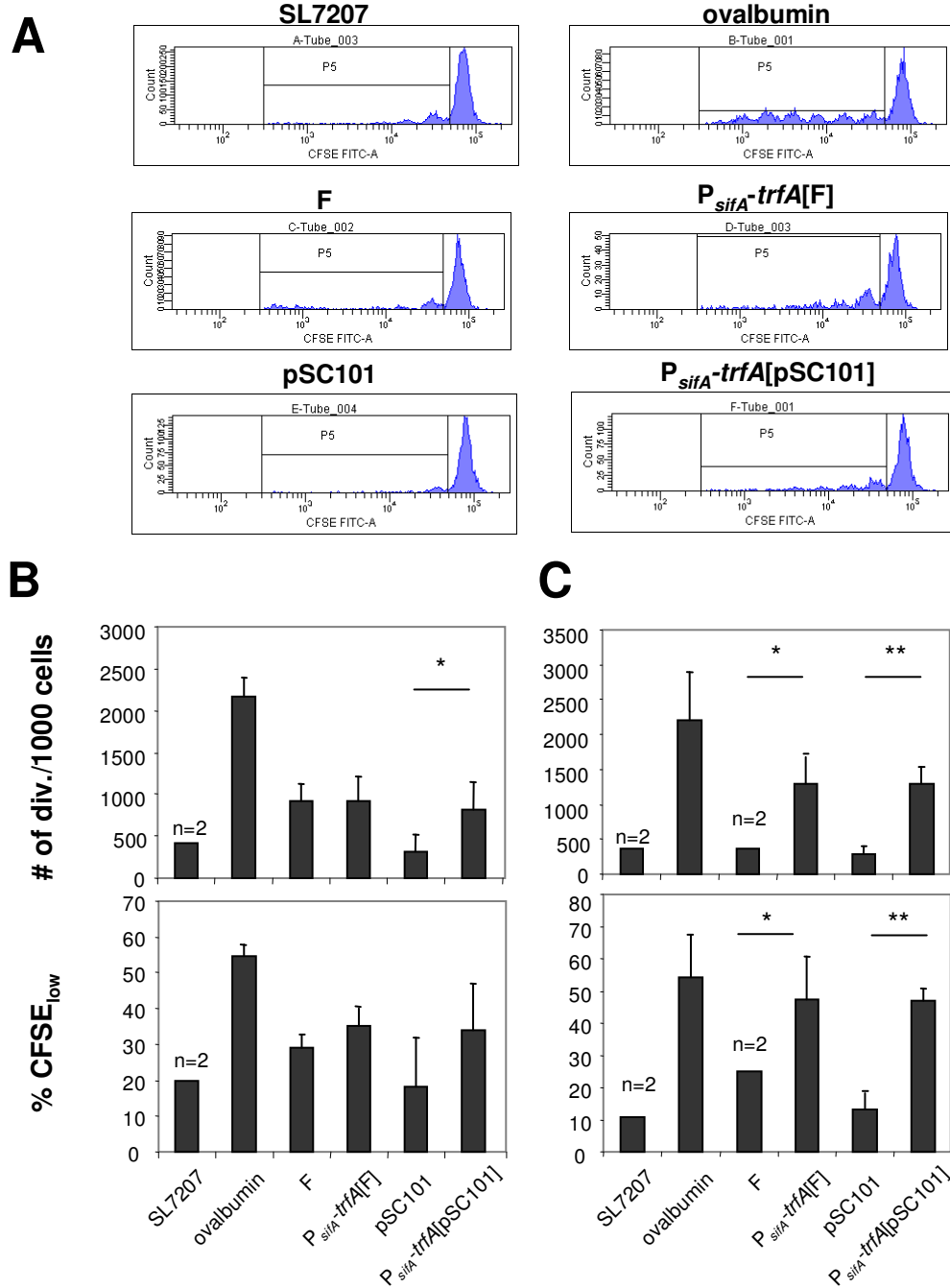


Fig. 3.12: Plasmid amplification leads to enhanced *in vivo* division of CFSE-labeled Ova₃₂₃₋₃₃₉-specific T cells. Chimeric mice were immunized i.v. with 5×10^5 cfu of the indicated strains or orally with 15 mg ovalbumin. 7 d p.i., antigen-specific T cells were analyzed via flow cytometry. (A) Representative histograms for MLN for one mouse per group gated on CD4⁺ and tgTCR positive lymphocytes. Number of cell divisions per 1000 tg T cells and percentages of cells with low fluorescence in MLN (B) and spleen (C). Unless otherwise indicated, averages for three mice per group (mean + s.d.) are shown (* $p < 0.05$; ** $p < 0.001$). Plasmid constructs are indicated with the first origin of replication only: F = pAEN51; pSC101 = pAEN61. Strains with inducible amplification of the expression plasmid are indicated as P_{sifA}-trfA. Experiment was performed twice with similar results.

As seen in Fig. 3.12 A, the fluorescence of CFSE labeled transgenic T cells decreased stronger in case of strains with inducible plasmid amplification and more cell divisions occurred apparently. This was confirmed when the number of cell divisions per 1000 specific T cells and the percentage of cells with low fluorescence intensity was calculated. All strains harboring P_{sifA} -*gfp_ova* expression plasmids exhibited an indicative decrease in fluorescence in spleen and MLN compared to the negative control (Fig. 3.12 B and C). Additionally, the number of cell divisions and percentage of CFSE_{low}-cells in spleen was significantly higher when the recombinant strains were able to amplify the expression plasmids *in vivo* (Fig. 3.12 C). Interestingly, no difference in the generation of the T helper cell response could be observed between the F plasmid and the pSC101 plasmid, although the latter is amplified twice as high. Probably, the higher stability of the system with the F plasmid replicon compensated the lower final copy number.

Taken together, *in vivo* amplification of low-copy number expression plasmids in *Salmonella* vectors leads to stronger CD4⁺ T cell responses after intravenous administration. This remains to be confirmed in an oral immunization scheme.

3.1.5.2 Generation of cytotoxic T cells

Thus far, the particular nature of the antigen only tested for induction of CD4⁺ T helper cells. In order to also analyze the CD8⁺ T cell response, a part of the listeriolysin O (*hly*) gene (amino acids 49-361) was placed under control of P_{sifA} and incorporated into amplifiable plasmids with the F plasmid or pSC101 replicon, generating plasmids pAEN98 or pAEN95, respectively (see Fig. 2.7). The antigenic fragment encodes a non-hemolytic variant of the pore-forming toxin LLO of *L. monocytogenes* that contains the MHC class I (H-2K^d) restricted T cell epitope LLO₉₁₋₉₉ (Pamer et al. 1991). This antigen is therefore suitable to measure the induction of CD8⁺ T cell responses.

To evaluate whether exchange of the antigen altered the pattern of plasmid amplification and antigen expression, strains SL7207 and SL7207:: P_{sifA} -*trfA* carrying pAEN95 or pAEN98 were grown in LB and induction medium and submitted to Western Blot analysis. Using SL7207 as carrier of the plasmids, protein expression was upregulated in induction medium due to the conditional promoter P_{sifA} (Fig. 3.13). A higher antigen expression could be observed when the plasmids were simultaneously amplified in strain SL7207:: P_{sifA} -*trfA*. Indeed, the LLO₄₉₋₃₆₁ containing plasmids were approximately

amplified up to 50 (F plasmid replicon) and 100 (pSC101 replicon) copies (data not shown) confirming the data obtained with *gfp_ova* as antigen.

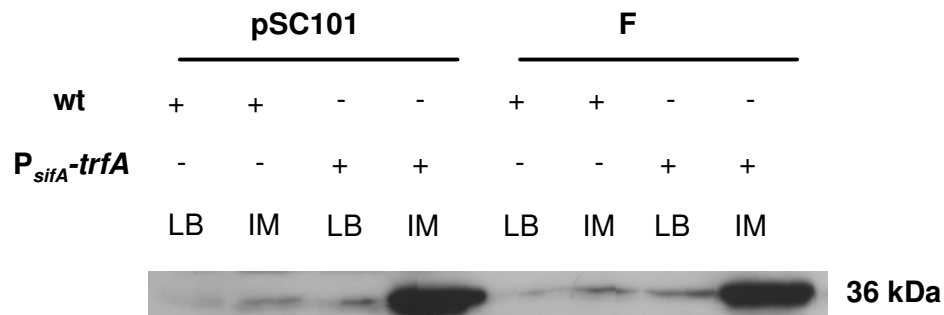


Fig. 3.13: Antigen expression and plasmid amplification with LLO₄₉₋₃₆₁ as antigen. Whole lysates of strains SL7207 and SL7207::P_{sifA}-trfA grown under non-inducing (LB) and inducing conditions (IM) were analyzed by Western Blot using a polyclonal antibody to LLO. The bacteria were transformed with P_{sifA}-LLO encoding amplifiable plasmids, which carry the F plasmid or pSC101 replicon to maintain the minimal-copy state.

To test whether the stability of the amplified plasmids could be influenced by the choice of antigen, the colonization abilities of strains expressing LLO₄₉₋₃₆₁ were also characterized. This had no influence on colonization efficiency and plasmid stability (data not shown).

Then, it was tested whether *in vivo* inducible plasmid amplification augments the generation of cytotoxic T cells. To this end, BALB/c mice were immunized orally with three doses of 1×10^9 cfu recombinant Salmonella expressing LLO₄₉₋₃₆₁ in 14 day intervals and the cytotoxic responses measured by an *in vivo* killer assay 10 d after the last immunization. Fig. 3.14 A represents a typical histogram. In naïve control mice, no cytotoxic reaction occurs such that untreated (low CFSE) and LLO₉₁₋₉₉ sensitized cells (high CFSE) remain at their initial 50:50 relationship. In positive control mice immunized once with *Listeria monocytogenes*, LLO₉₁₋₉₉ sensitized cells are killed, which can be calculated as specific killing (Fig. 3.14 A). In the group immunized with SL7207::P_{sifA}-trfA harboring P_{sifA}-LLO expression plasmid pAEN98 (F plasmid replicon), one mouse out of three exhibited cytotoxic activity (28% specific killing). Both mice immunized with SL7207::P_{sifA}-trfA harboring P_{sifA}-LLO expression plasmid pAEN95 (pSC101 plasmid replicon) mounted a specific cytotoxic T cell response (Fig. 3.14 B). However,

reproduction of these results failed. In such experiments, mice were immunized with SL7207 and SL7207::P_{sifA}-trfA carrying the P_{sifA}-LLO expression plasmids in order to assess the influence of plasmid amplification. No specific killing could be detected after three oral immunizations. Likewise, mice immunized thrice i.v. with $0.5-1 \times 10^5$ cfu of the same recombinant strains did not mount a cytotoxic response against the antigen. Obviously, the high antigen expression induced in the recombinant Salmonella strains is not sufficient to circumvent the problem of antigen delivery to the cytosol of infected cells and subsequent MHC class I presentation.

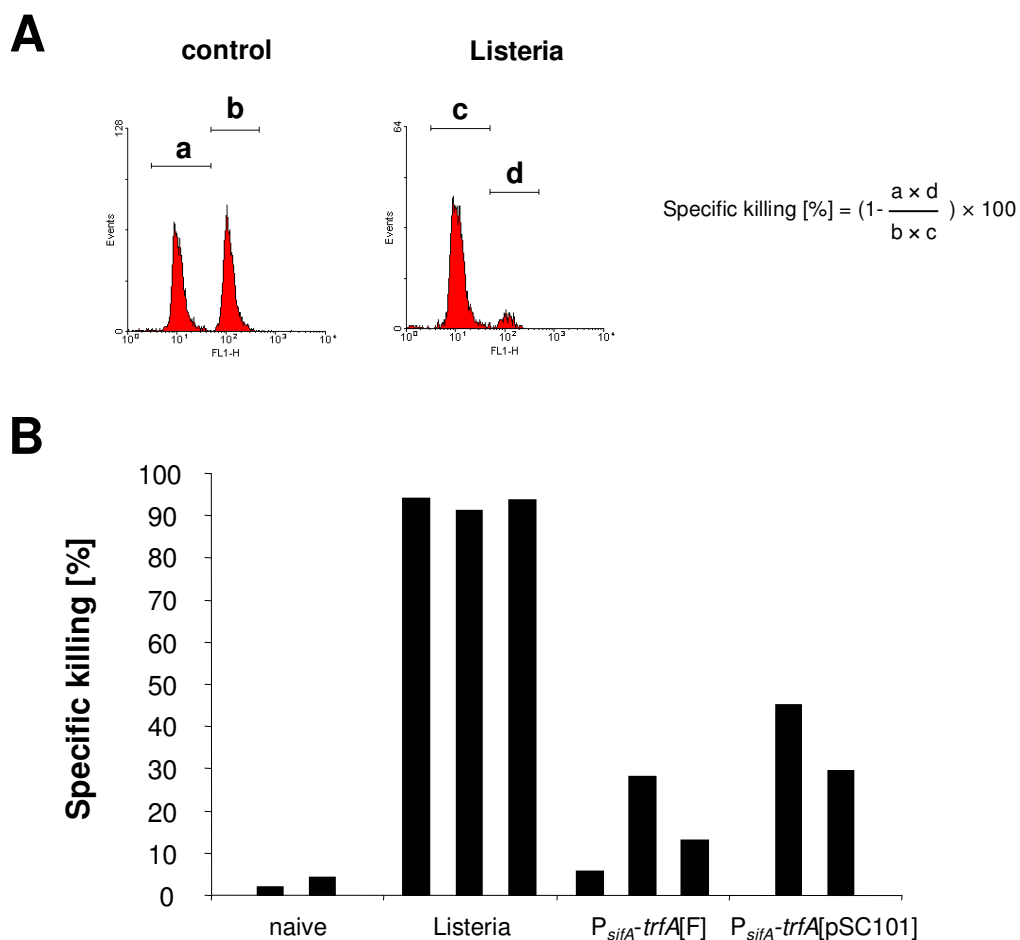


Fig. 3.14: *In vivo* cytotoxicity assay after immunization with LLO₄₉₋₃₆₁ expressing Salmonella.

(A) Representative histograms for naïve control and Listeria-immunized mice and formula used for calculating specific killing. (B) Specific killing determined in individual mice that received a single sublethal dose of Listeria or three doses of recombinant Salmonella. Plasmid constructs are indicated with the first origin of replication only: F = pAEN98; pSC101 = pAEN95. P_{sifA}-trfA indicates strains with inducible amplification of the expression plasmids.

3.1.6 Simultaneous *in vivo* inducible plasmid amplification and remote control of bacterial lysis by L-arabinose

The Salmonella-mediated delivery of antigens or DNA vaccines requires liberation of the proteins or plasmids after invasion of APC. A system for programmed lysis of the bacterial carrier might be suitable for such a purpose. Therefore, the lysin *gene E* of the bacteriophage ϕ X174 was placed under control of the L-arabinose inducible promoter P_{BAD} . It had been shown before that already 100 molecules of the lysin encoded by *gene E* lead to loss of bacterial viability (Maratea et al. 1985). The construct was integrated as a single copy into the chromosome of SL7207 together with the fragment P_{sifA} -*trfA* for amplification (strain SL7207::AEN106 in Fig. 2.8). This strain should mediate plasmid amplification and in addition, be prone to L-arabinose inducible lysis (Fig. 3.15).

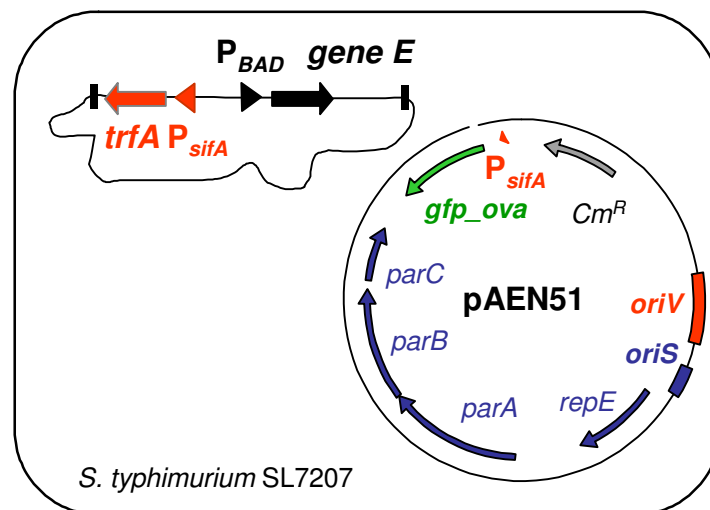


Fig. 3.15: Strategy for simultaneous *in vivo* inducible plasmid amplification and remote control of bacterial lysis by L-arabinose. Upon activation of P_{sifA} , the chromosomally integrated replication protein TrfA is expressed and mediates amplification of pAEN51 from the second replicon, *oriV*. Additionally, the strain SL7207::AEN106 harbors a chromosomal cassette encoding lysin *gene E* under control of the L-arabinose inducible promoter P_{BAD} .

To test whether the additional lysis cassette interferes with plasmid amplification, strain SL7207::AEN106 was transformed with plasmid pAEN51 and GFP expression was analyzed in comparison to SL7207:: P_{sifA} -*trfA*. No differences in the ability to mediate plasmid amplification and antigen expression were observed between the two strains in the

absence of L-arabinose (Fig. 3.16 A). This indicates that the integration of the lysis cassette does not affect the expression of the replication protein TrfA.

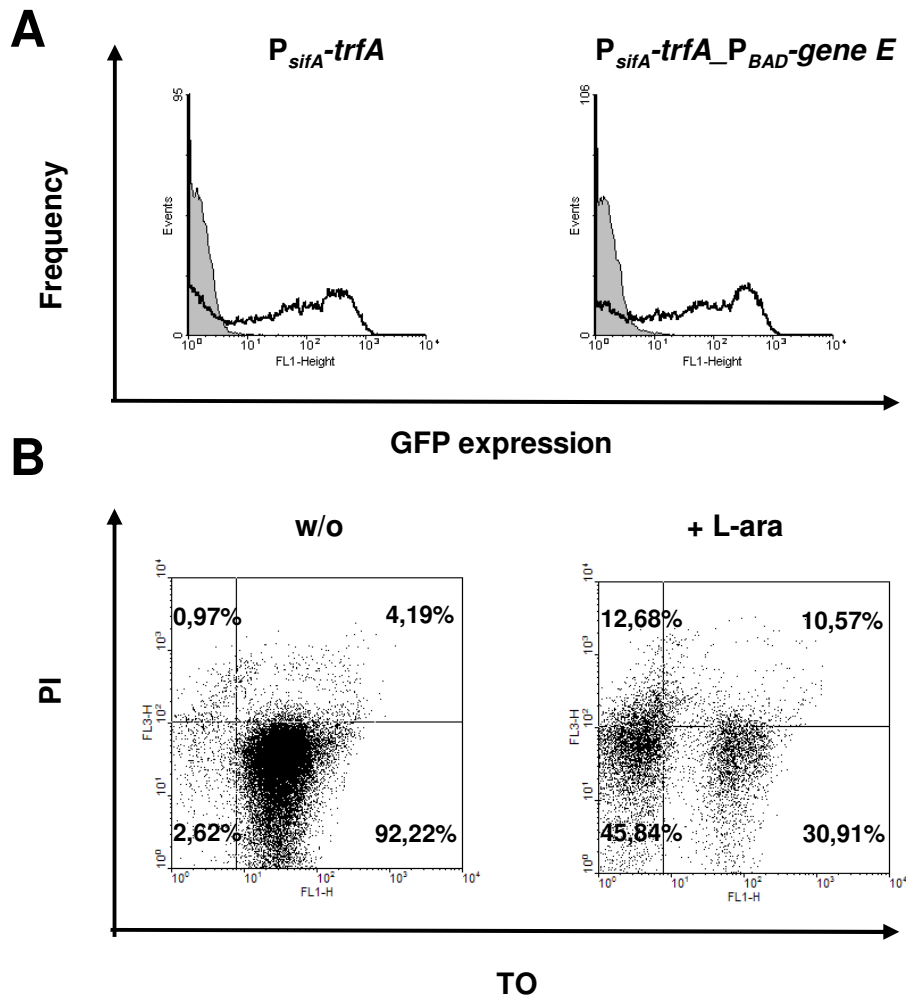


Fig. 3.16: Simultaneous inducible plasmid amplification and remote control of bacterial lysis by L-arabinose in culture. (A) GFP expression of indicated chromosomal integration derivatives transformed with plasmid pAEN51 grown in LB (shaded curve) or induction medium (thick lines) in the absence of L-arabinose. (B) SL7207::AEN106[pAEN51] was grown in LB in the absence or presence of 0,1% L-arabinose for 4 h and subjected to live/dead staining and subsequent flow cytometric analysis. Comparable results could be obtained with strains transformed with pAEN61 (data not shown).

However, upon addition of the inducer, visible lysis occurred in cultures of strain SL7207::AEN106 containing chromosomally integrated $P_{BAD}-gene\ E$. This could be confirmed by applying live/dead staining after culturing the strain in the absence and presence of L-arabinose (Fig. 3.16 B). The assay is based on staining the bacterial DNA with the membrane-permeable dye thiazole orange (TO) and the membrane-impermeable

dye propidium iodide (PI). Without inducer, bacteria were mainly stained by TO and thus were viable, whereas bacteria became PI positive after adding L-arabinose since the membranes were damaged by induced *gene E*. In addition, an unstained bacterial population appeared after L-arabinose induced lysis. This probably reflected bacteria that had already released their content into the surrounding media. Taking together, an additional lysis cassette did not interfere with tight control of plasmid amplification and antigen expression but allowed remote control of bacterial viability by an easily available inducer. It remains to be established whether such a vaccine strain with *in vivo* amplifiable plasmid replication and antigen expression augments the generation of CD4⁺ T-helper cell responses upon lysis of the bacterial carrier strain.

3.1.7 Non-invasive *in vivo* imaging of P_{sifA} induction

A recent study described the activation of SPI-2 promoters P_{sseA}, P_{spiC} and P_{ssaG} already in the murine intestine during initial stages of Salmonella pathogenesis (Brown et al. 2005). This is contradictory to the established model that argues for exclusive intracellular expression of SPI-2. Activation of P_{sifA} prior to penetration of the intestinal epithelium could lead to impaired invasion efficiency of strains carrying the chromosomal integration P_{sifA-trfA} and/or P_{sifA} expression plasmids. However, the promoter P_{sifA} was not tested by this group. Therefore, this matter was addressed using bioluminescence *in vivo* imaging.

The amplifiable plasmid pAEN102 was constructed that contains the *lux* operon of the bacterium *Photobacterium luminescens* (Meighen and Szittner 1992) as reporter (Fig. 3.17). Expression of this reporter results in continuous light emission without the need of exogenous substrate and allows simple monitoring of bacteria in live mice (Contag et al. 1995).

The parental strain SL7207 and the chromosomal integration derivative P_{sifA-trfA} were transformed with plasmid pAEN102 and tested for light emission *in vitro*. As expected, in induction medium, strain P_{sifA-trfA} generated more bioluminescence than strain SL7207 due to amplification of the expression plasmid (data not shown). Importantly, both strains exhibited similar levels of background light activity in LB medium (data not shown). This confirmed again the tight control of P_{sifA} under non-inducing conditions.

To follow the *in vivo* induction of P_{sifA} , mice were infected orally with SL7207 or P_{sifA} -*trfA* carrying plasmid pAEN102 and monitored for 4 h using the supercooled CCD camera system IVIS-200 (Xenogen). As control, recombinant SL7207 harboring plasmid pSL1 (Winson et al. 1998) were administered. Plasmid pSL1 offers weak constitutive expression of the *lux* genes. Already 30 min after oral administration of recombinant P_{sifA} -*trfA*, a strong bioluminescence signal was detected in the gut region (Fig. 3.18 A). In contrast, SL7207 harboring pAEN102 emitted light of lower intensity (Fig. 3.18 A and B). Such difference can only be attributed to plasmid amplification induced by activated P_{sifA} . This clearly argues for activation of the promoter in the gut lumen prior to breaching of the intestinal barrier.

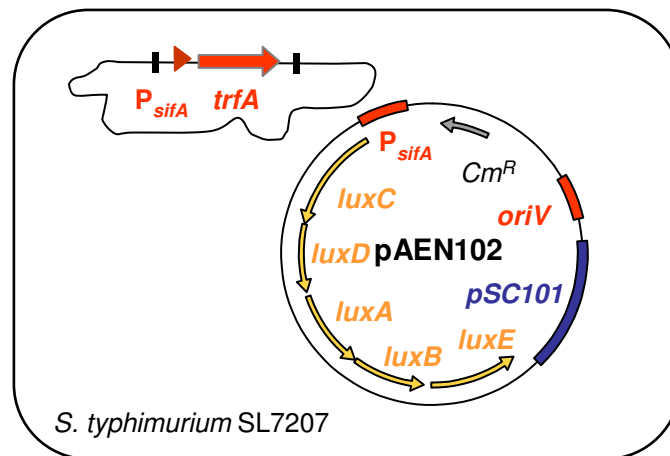


Fig. 3.17: Strategy for non-invasive *in vivo* imaging of P_{sifA} induction. Upon activation of P_{sifA} , the promoter drives the expression of the *lux* operon. The reporter is encoded on pAEN102 which is maintained at 5 copies per bacterial cell. Amplified replication of pAEN102 from *oriV* is mediated by TrfA, whose expression is also activated by P_{sifA} .

In all strains, light emission diminished from 30 min to 4 h p.i. (Fig. 3.18 A and B). This might be due to a change in the localization of the bacteria relative to the surface of the mouse. Alternatively, bacterial shedding after movement through the intestinal tract via feces could be the reason.

Activation of P_{sifA} very early in the intestine most likely lowers the invasion efficiency of recombinant Salmonella. However, when mice infected with P_{sifA} -*trfA* carrying plasmid pAEN102 were sacrificed 5 h p.i., *ex vivo* images of the intestine revealed weak

bioluminescence signals localized to areas of the PP (data not shown). This indicates that some bacteria are able to penetrate the intestine and reach immune inductive sites of lymphoid tissues as already shown in the colonization studies. Nevertheless, this demonstrates the need to explore also alternative promoters for plasmid amplification, like promoters that are controllable by external inducers.

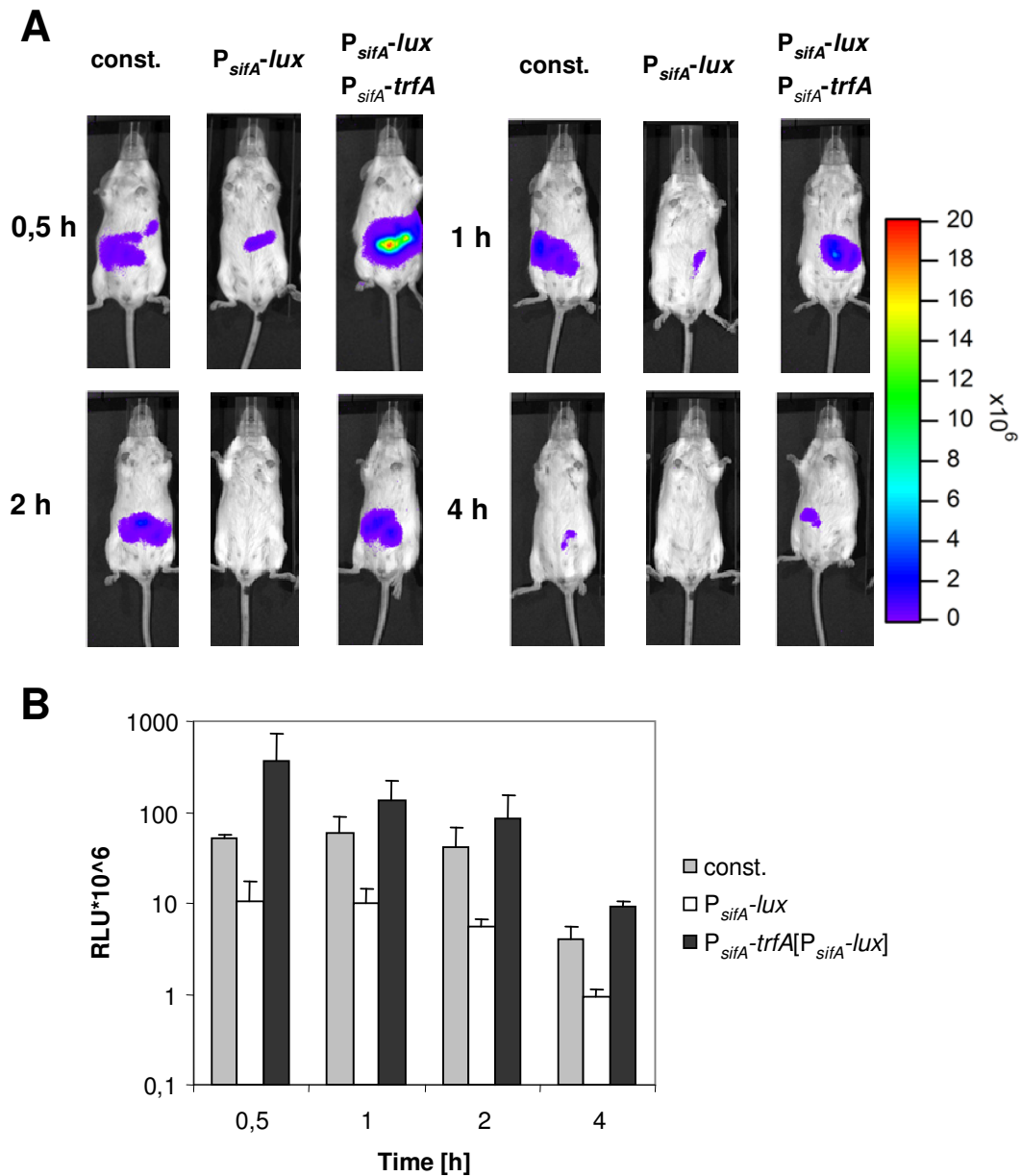


Fig. 3.18: Non-invasive *in vivo* imaging of bacterial bioluminescence. Mice were infected orally with 2×10^9 cfu of *S. typhimurium* harboring either the inducible P_{sifA}-lux expression plasmid pAEN102 or plasmid pSL1 with constitutive lux expression. The strain with inducible amplification of the expression plasmid is indicated as P_{sifA}-trfA. (A) Images of individual anaesthetized mice were acquired at indicated time points. (B) The intensity of bioluminescence emission was quantified for all mice of each group. Values represent the average for three mice per data point (mean + s.d.).

3.2 Remote control of plasmid amplification by L-arabinose

The use of *in vivo* inducible promoters as mediators for plasmid amplification was considered suitable for immunizations. An alternative application might be the use of *Salmonella* that colonize tumors for therapy. Thus, it might be advantageous to control bacteria at a defined time *in vivo* by administration of an inducer. For that purpose, it was decided to concentrate on the L-arabinose inducible promoter P_{BAD} and regulator AraC from the arabinose operon of *Escherichia coli*, which was applied already before for induced bacterial lysis. The *araC*- P_{BAD} system is often used for controlled gene expression in *E. coli* and other bacteria, as it offers regulatable control in the presence and tight control in the absence of inducer (Guzman et al. 1995). Therefore, plasmid amplification was placed under control of P_{BAD} and the amplification system was characterized in medium, in cell culture and subsequently *in vivo* in a mouse tumor model.

3.2.1 Amplification of a single copy plasmid controlled by plasmid-encoded P_{BAD}

Expression plasmids pAEN9 (see Fig. 2.1) and pAEN38 were constructed to investigate the induction of plasmid amplification under remote control. These plasmids are equipped with the F plasmid derived single-copy maintenance system *oriS-repE-parABC* and a second replicon based on the origin of replication *oriV* and the replication protein TrfA, whose expression is controlled by P_{BAD} . Moreover, the plasmids encode reporter genes *gfp_ova* (pAEN9) and firefly luciferase (pAEN38) under control of the weak constitutive promoter P_{bla} . *S. typhimurium* SL7207 was transformed with these vectors and cultures of such transformants were induced with different concentrations of L-arabinose for 4 h. As expected, without addition of L-arabinose, the plasmids were present at a single copy state (Fig. 3.19 A). Both reporter plasmids were amplified to comparable numbers depending on the concentration of L-arabinose in the growth medium. In concordance, plasmid amplification resulted in strong induction of GFP_ova and luciferase expression (Fig. 3.19 B and C).

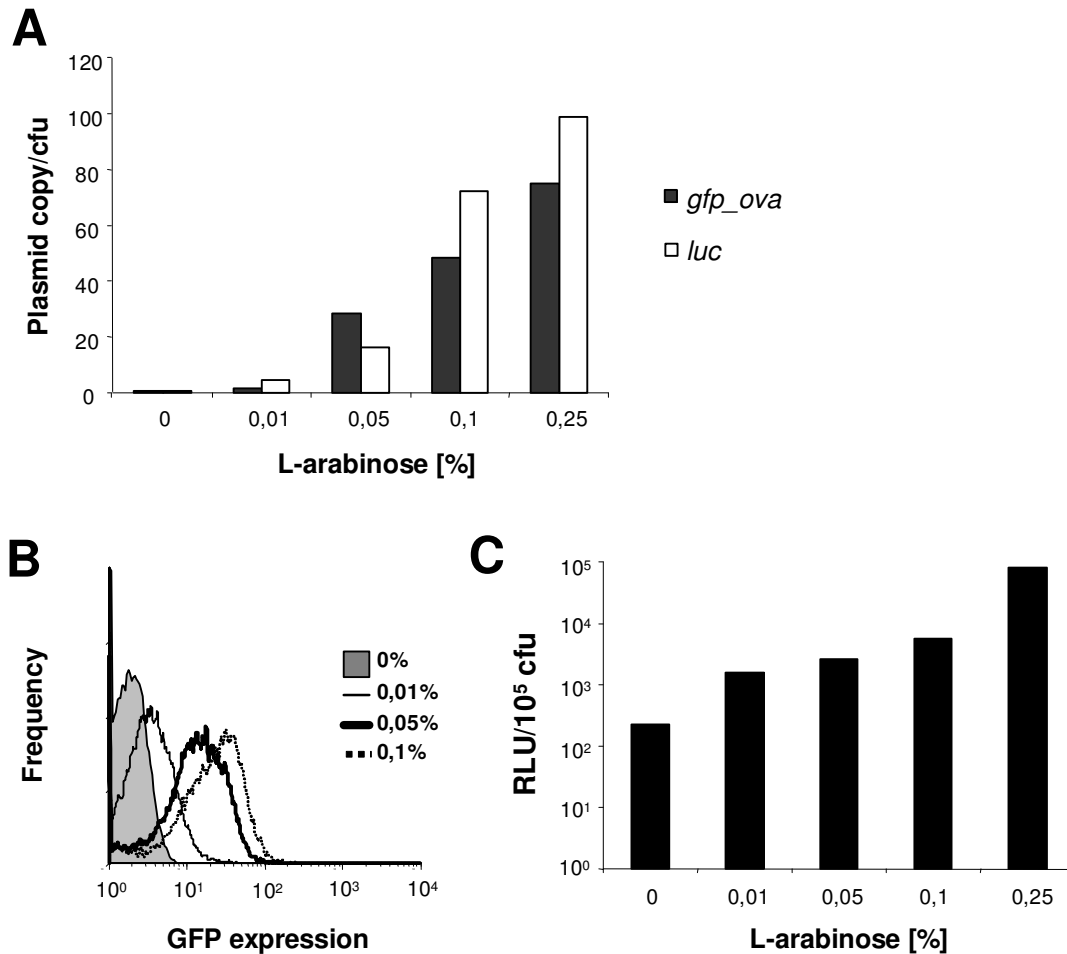


Fig. 3.19: Plasmid amplification of reporter plasmids after induction with L-arabinose in bacterial cultures. (A) SL7207 harboring pAEN9 (*gfp_ova*) or pAEN38 (*luc*) were induced with different concentrations of L-arabinose in LB medium and plasmid copy/cfu were determined 4 h after induction. (B) Bacteria harboring pAEN9 were induced as in a) and GFP expression was analyzed by flow cytometry. (C) Bacteria harboring pAEN38 were induced as in a) and enzymatic activity of luciferase was determined from lysates and calculated as relative light units (RLU) per 10⁵ cfu.

Subsequently, induced plasmid amplification was tested when *Salmonella* resided inside host cells. In case of the reporter GFP, plasmid pAEN17 was employed. This vector encodes the stable variant *gfpmut2* instead of degradable *gfp_ova* in order to allow accumulation of the reporter within the bacteria. Thereby, the sensitivity of detection via two color flow cytometry is enhanced. *S. typhimurium* SL7207 was transformed with expression plasmids pAEN17 and pAEN38 and J774-A1 mouse macrophages were infected with the transformants. Extracellular bacteria were killed with gentamycin and 3 h p.i. L-arabinose was added to the cultures at different concentrations. After incubation for 4 h, cells were lysed and bacteria analyzed for reporter gene expression. As shown in

Fig. 3.20 A and B, increasing concentrations of L-arabinose led to increased bacterial expression of the reporters, indicating a successful amplification of the expression plasmids. Furthermore, the number of GFP-expressing *Salmonella* above the background autofluorescence increased with rising concentrations of L-arabinose.

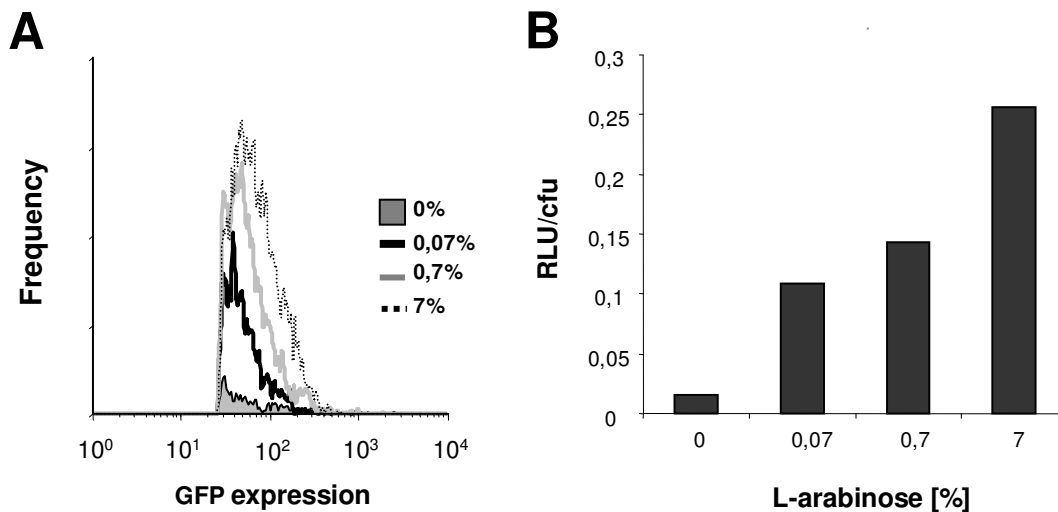


Fig. 3.20: Plasmid amplification of reporter plasmids after induction with L-arabinose after infection of mouse macrophages. SL7207 harboring pAEN17 (*gfp*) or pAEN38 (*luc*) were used to infect J774-A1 macrophages. 3 h p.i., L-arabinose at indicated final concentrations was added. (A) Bacterial GFP expression in cellular lysates was measured by two color flow cytometry 4 h after induction. (B) Luciferase expression by intracellular bacteria was determined as relative light units (RLU) per cfu 4 h after induction.

Having established that L-arabinose reaches bacteria residing in phagosomal compartments of macrophages, it was tested whether plasmid amplification could be also induced *in vivo*. Mice were infected intravenously with *S. typhimurium* SL7207 carrying the amplifiable GFP expression plasmid pAEN17 or luciferase expression plasmid pAEN38, respectively. Three days later, different amounts of L-arabinose were injected intraperitoneally and 6 h later reporter gene expression was determined in bacteria recovered from lysed spleen cells. Administration of L-arabinose resulted in increasing numbers of GFP-expressing *Salmonella* and increasing values of the median GFP fluorescence intensity with rising concentrations of the inducer (Fig. 3.21 A). Likewise, a strong induction of bacterial luciferase expression could be observed in spleens from infected mice after L-arabinose injection (Fig. 3.21 B). Based on these results, it was decided to administer the highest amount of L-arabinose (120 mg) and to concentrate on luciferase as reporter for the

experiments with tumor-targeted *Salmonella*. In contrast to GFP, the use of luciferase as reporter is not hampered by high background activity of mammalian cells and tissues.

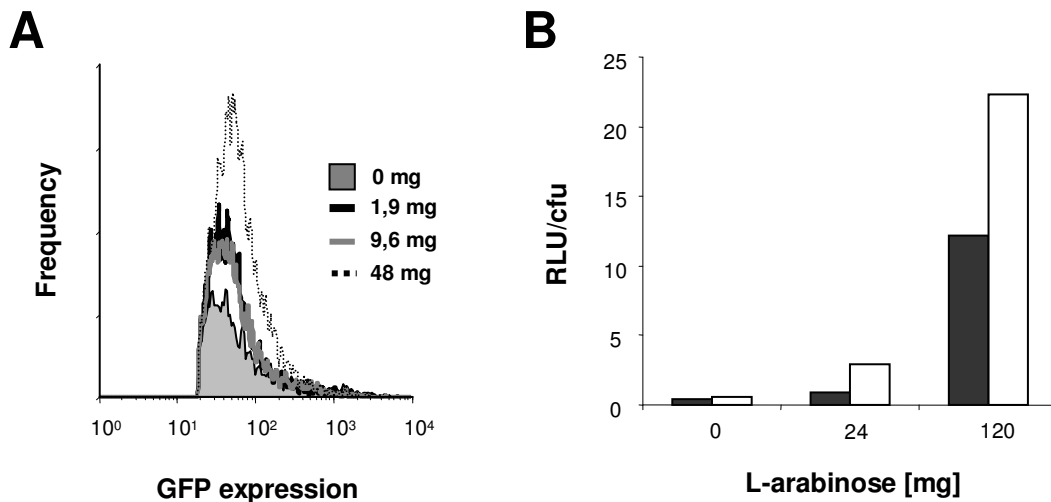


Fig. 3.21: Plasmid amplification of reporter plasmids after induction with L-arabinose after colonization of spleen. Mice were infected with 2×10^6 SL7207 harboring pAEN17 (*gfp*) or pAEN38 (*luc*). 3 d p.i., L-arabinose at indicated amounts was injected i.p.. (A) Bacterial GFP expression in splenic lysates was measured by two color flow cytometry 6 h after induction. Histograms represent individual mice. (B) Luciferase expression by intracellular bacteria was determined as relative light units (RLU) per cfu 6 h after induction. Bars represent individual mice.

3.2.2 Amplification of a single copy plasmid controlled by chromosomally integrated P_{BAD}

Thus far, the expression plasmids contained the replication elements of the F plasmid for single-copy maintenance and the P_{BAD} -*trfA*/*oriV* replicon for L-arabinose inducible plasmid amplification. For that reason, the size and complexity of those plasmids is rather large, which might impair plasmid stability *in vivo*. In order to reduce the plasmid size, the P_{BAD} -*trfA* cassette was integrated into the *Salmonella* chromosome generating the strain SL7207::AEN46 (see Fig. 2.8). This strain was transformed with the minimal plasmid pAEN35 that lacks the P_{BAD} -*trfA* cassette and harbors *gfp_ova* under control of the promoter P_{bla} . Cultures of such transformants were induced with different concentrations of L-arabinose for 4 h. Although the copy number of pAEN35 increased with rising concentrations of the sugar, it was amplified to a lesser extent compared to plasmid pAEN9 (Fig. 3.22 B vs. Fig. 3.19 A). Likewise, a lower induction of GFP_{ova} expression

could be observed (Fig. 3.22 B vs. Fig. 3.19 B). Obviously, chromosomal expression of the replication protein TrfA under control of P_{BAD} was not sufficient for amplification to higher copy numbers. This was also confirmed when strain SL7207::AEN44 (see Fig. 2.8), a chromosomal integration derivative of P_{BAD} -gfp, was tested for reporter gene expression after L-arabinose induction *in vitro*. Only a slight increase of fluorescence intensity could be observed under inducing conditions (Fig. 3.22 A), showing that P_{BAD} is a weaker promoter compared to the *in vivo* inducible promoters tested in the first part of this work (see Fig. 3.1).

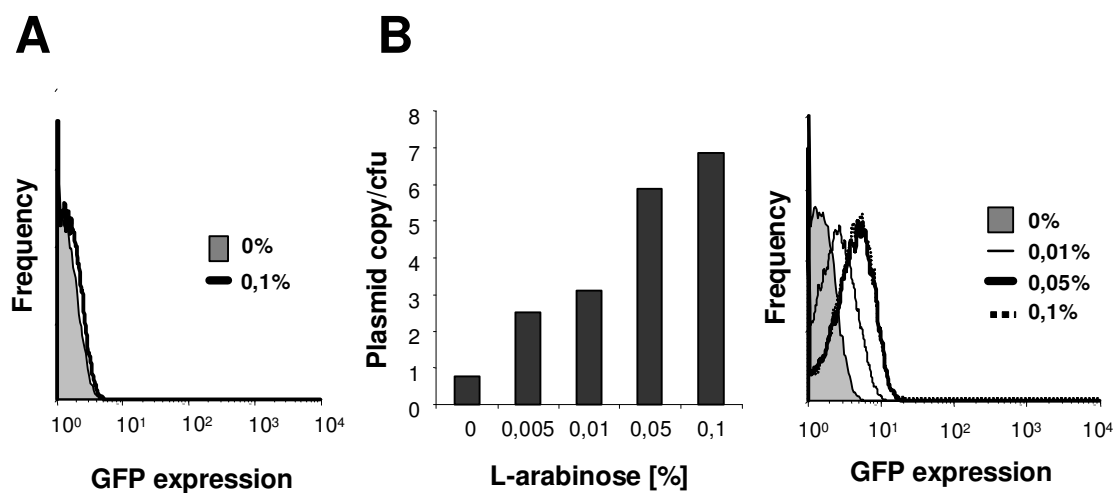


Fig. 3.22: Gene expression from chromosomally located P_{BAD} is not sufficient for strong plasmid amplification. (A) Flow cytometric detection of *in vitro* GFP expression of SL7207::AEN44 (P_{BAD} -gfp) grown for 4 h under non-inducing conditions (LB w/o L-arabinose, shaded curve) and inducing conditions (LB with 0,1% L-arabinose, black lines) (B) SL7207::AEN46 (P_{BAD} -trfA) harboring pAEN35 was induced with different concentrations of L-arabinose in LB medium and plasmid copy/cfu and GFP expression were determined 4 h after induction.

Taking together, a reduction of plasmid size (11,3 kb vs. 8,4 kb) was achieved by removing the P_{BAD} -trfA cassette from plasmid pAEN9. However, the minimal plasmid pAEN35 could only be amplified from 1 to 8 copies by chromosomally integrated P_{BAD} -trfA. Therefore, it was decided to control plasmid amplification by plasmid-encoded P_{BAD} -trfA in further experiments.

3.2.3 Remote control of simultaneous amplification of plasmid replication and protein expression

The same strategy for the enhancement of copy number and gene expression described in section 3.1.3 was also employed for remote control by L-arabinose. The luciferase expression plasmid pAEN59 (see Fig. 2.2) was constructed, which is derived from plasmid pAEN38 but harbors the pSC101 replicon instead of the F plasmid replicon to maintain the minimal-copy state. Transformants of SL7207 carrying these plasmids were induced *in vitro* with different concentrations of L-arabinose for 4 h. Amplification of pAEN59 resulted in approximately five times higher copy number than pAEN38 (Fig. 3.23 A). Consequently, a higher luciferase expression could be achieved (Fig. 3.23 B). Importantly, both plasmids were stably propagated without antibiotic selection *in vitro* (data not shown).

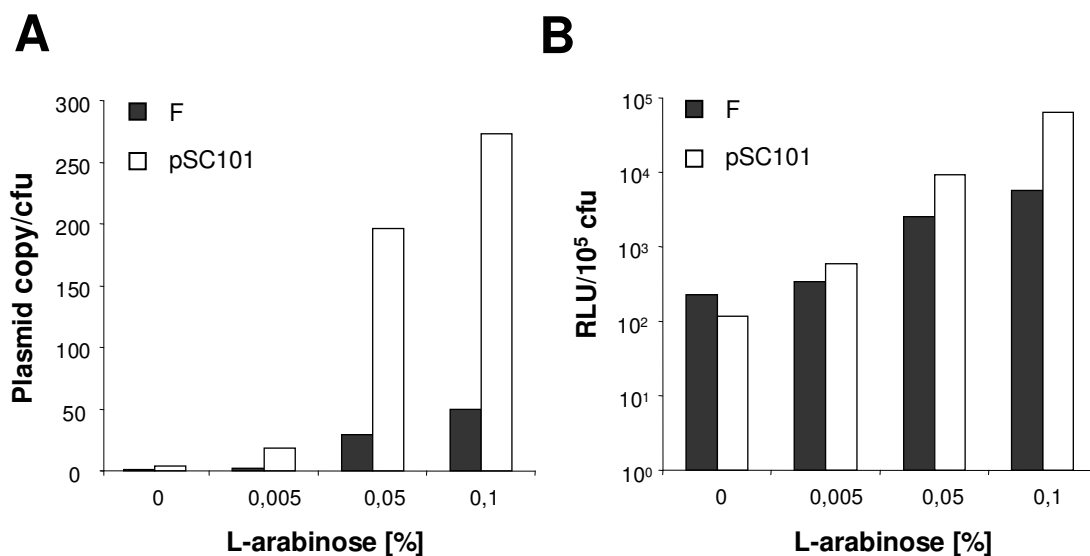


Fig. 3.23: *In vitro* plasmid amplification of luciferase expression plasmids with different minimal copy replicons after induction with L-arabinose. (A) SL7207 harboring pAEN38 (F plasmid replicon) or pAEN59 (pSC101 replicon) were induced with different concentrations of L-arabinose in LB medium and plasmid copy/cfu were determined 4 h after induction. (B) Bacteria were induced as in a) and enzymatic activity of luciferase was determined from lysates and calculated as relative light units (RLU) per 10⁵ cfu.

As an additional way to enhance reporter gene expression, luciferase expression was placed under control of P_{BAD} instead of the weak constitutive P_{bla} promoter resulting in plasmid pAEN64 (see Fig. 2.3). SL7207 was transformed with this plasmid and the transformants were induced for 4 h with different concentrations of L-arabinose. As expected, simultaneous induction of plasmid amplification and protein expression by P_{BAD}

resulted in increased luciferase expression compared to the combination of L-arabinose inducible plasmid amplification and constitutive luciferase expression (Fig. 3.24). Moreover, a lower background expression was observed under non-inducing conditions. Additionally, plasmid pAEN110 was constructed, which is a derivative of pAEN64 lacking the P_{BAD} -*trfA* cassette. This plasmid is not amplifiable by L-arabinose and the measured luciferase expression can be attributed entirely to protein expression driven by P_{BAD} . Consequently, luciferase expression is reduced by 1 log in bacteria carrying pAEN110 instead of pAEN64 (Fig. 3.24). This points out that plasmid amplification contributed indeed to the strong luciferase expression.

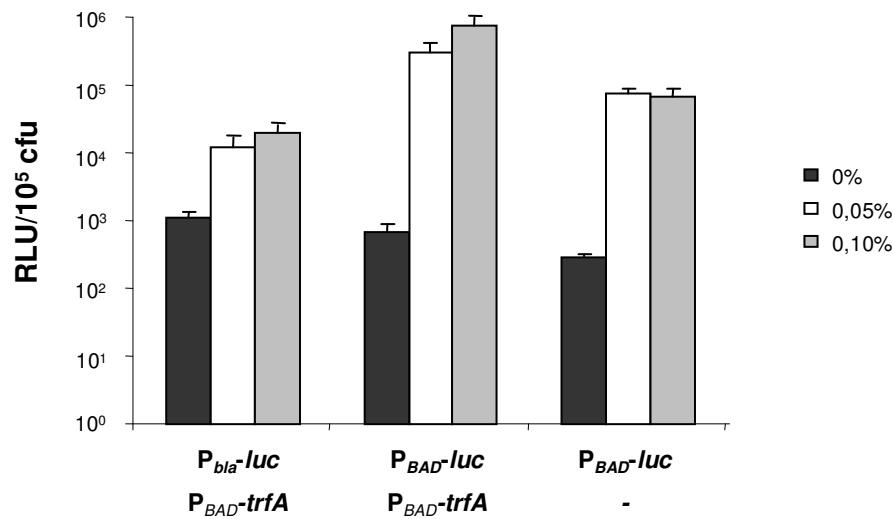


Fig. 3.24: Simultaneous plasmid amplification and protein expression by L-arabinose. SL7207 harboring pAEN59 (P_{BAD} -*trfA*/ P_{bla} -*luc*), pAEN64 (P_{BAD} -*trfA*/ P_{BAD} -*luc*) and pAEN110 ($-P_{BAD}$ -*luc*) were induced with different concentrations of L-arabinose in LB medium and enzymatic activity of luciferase was determined from lysates and calculated as relative light units (RLU) per 10⁵ cfu. Values represent the average for three cultures per data point (mean + s.d.).

In summary, a higher copy number could be achieved under inducing conditions by exchanging the origin of replication that determines the initial minimal-copy number. Furthermore, simultaneous control of protein expression and plasmid amplification by P_{BAD} resulted in higher reporter gene expression and lower background.

3.2.4 Remote control of plasmid amplification in tumor-colonizing SL7207

Before testing the control of plasmid amplification in tumor-colonizing *S. typhimurium* SL7207 *in vivo*, it was important to determine how L-arabinose accumulates in the circulation of mice to induce the P_{BAD} promoter. Therefore, 120 mg L-arabinose were injected intraperitoneally and the concentration of the sugar was determined. Fig. 3.25 shows that a high but transient concentration of L-arabinose could be found in the blood of mice. This concentration should be sufficient to induce bacterial gene expression under the control of P_{BAD} . The decrease after 4-6 h is probably due to degradation by host enzymes (Seri et al. 1996).

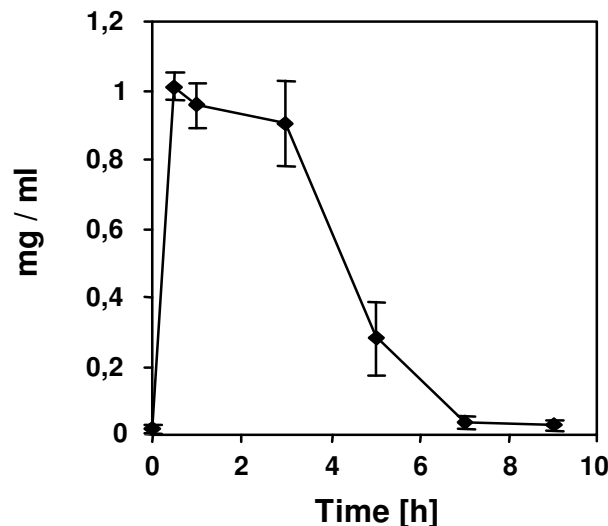


Fig. 3.25: Accumulation of L-arabinose after intraperitoneal administration of 120 mg sugar. Concentration of L-arabinose in blood plasma was measured at consecutive time points. Values represent the average for three mice per data point (mean + s.d.); experiment was performed three times with similar results.

Then, SL7207 carrying the amplifiable P_{bla} -luciferase expression plasmid pAEN38 were injected intravenously into mice bearing a subcutaneous CT26 tumor of 5-8 mm diameter. Seven days later, L-arabinose was injected intraperitoneally and colonization as well as bacterial luciferase expression was determined in tumor, liver and spleen. In individual mice, a 10-100 fold enrichment of bacteria in tumors was observed (Fig. 3.26 A). This was not as dramatic as previously observed with other *S. typhimurium* strains (Bermudes et al. 2002) and might be due to the strain SL7207 used here, which was not particularly

optimized for tumor-targeting. The accumulation ratio was rather variable during all tumor experiments, although all tumors were colonized. Moreover, the accumulation ratio did not increase from 7 to 14 days after injection of the bacteria (data not shown). Administration of L-arabinose resulted in increased reporter expression in bacteria from all three tissues (Fig. 3.26 B). This is due to induction of plasmid amplification by P_{BAD} . Interestingly, without induction, a higher plasmid stability was observed in tumors (94 +/- 6%) compared to liver (62 +/- 8%) and spleen (60 +/- 11%) (Fig. 3.26 C).

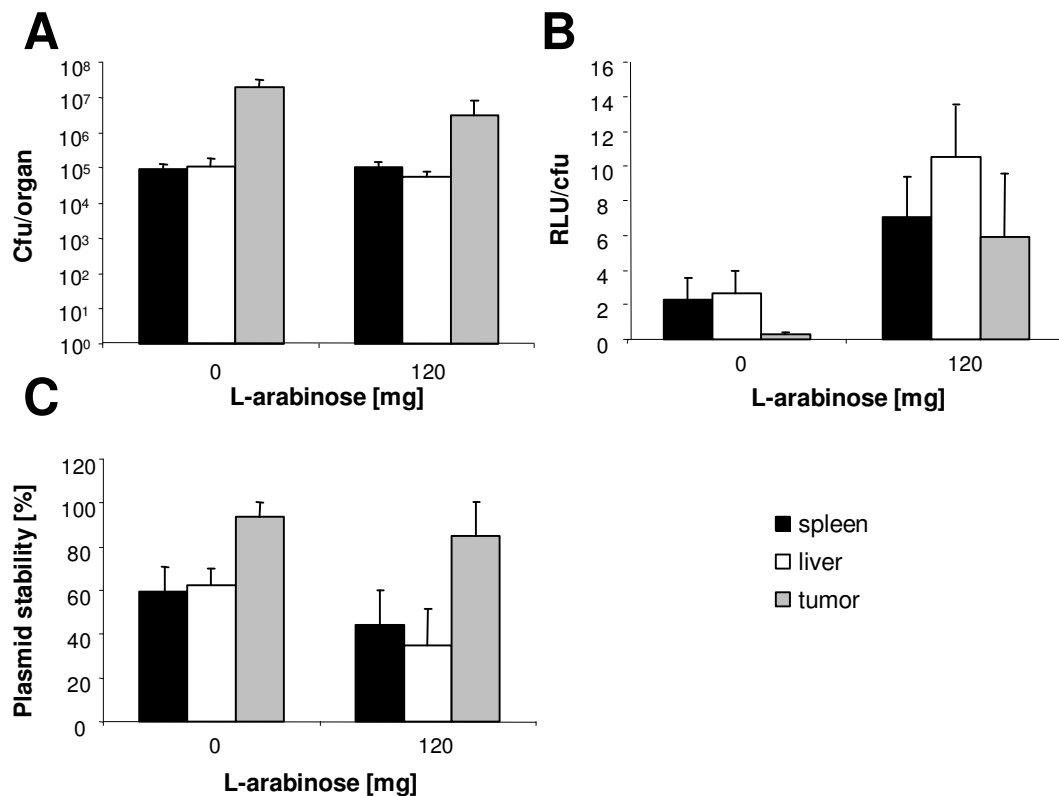


Fig. 3.26: L-arabinose induced plasmid amplification in tumor-bearing BALB/c mice. 7 d p.i. of 5×10^5 SL7207 carrying pAEN38, groups of mice were injected intraperitoneally with 0 and 120 mg of L-arabinose for 6 h. (A) Colonization of host tissue as determined from plating of tissue homogenates. (B) Bacterial luciferase expression was determined in tissue lysates and calculated as relative light units (RLU) per CFU. (C) Stability of pAEN38 in recombinant SL7207. Values represent the average for three mice per data point (mean + s.d.); experiment was performed twice with similar results.

Although plasmid pAEN38 was stably retained in SL7207 *in vitro*, it was not fully stable *in vivo* (values ranged from 2-80% plasmid stability 14 d after bacterial injection, data not shown). Therefore, experiments were also performed with SL7207 carrying pAEN59, which harbors the pSC101 replicon instead of the F plasmid replicon. In this case, a similar

bacterial colonization could be observed and the plasmid was stably retained in approximately 100% and 70% of the bacteria 7 days and 14 days after injection, respectively (data not shown).

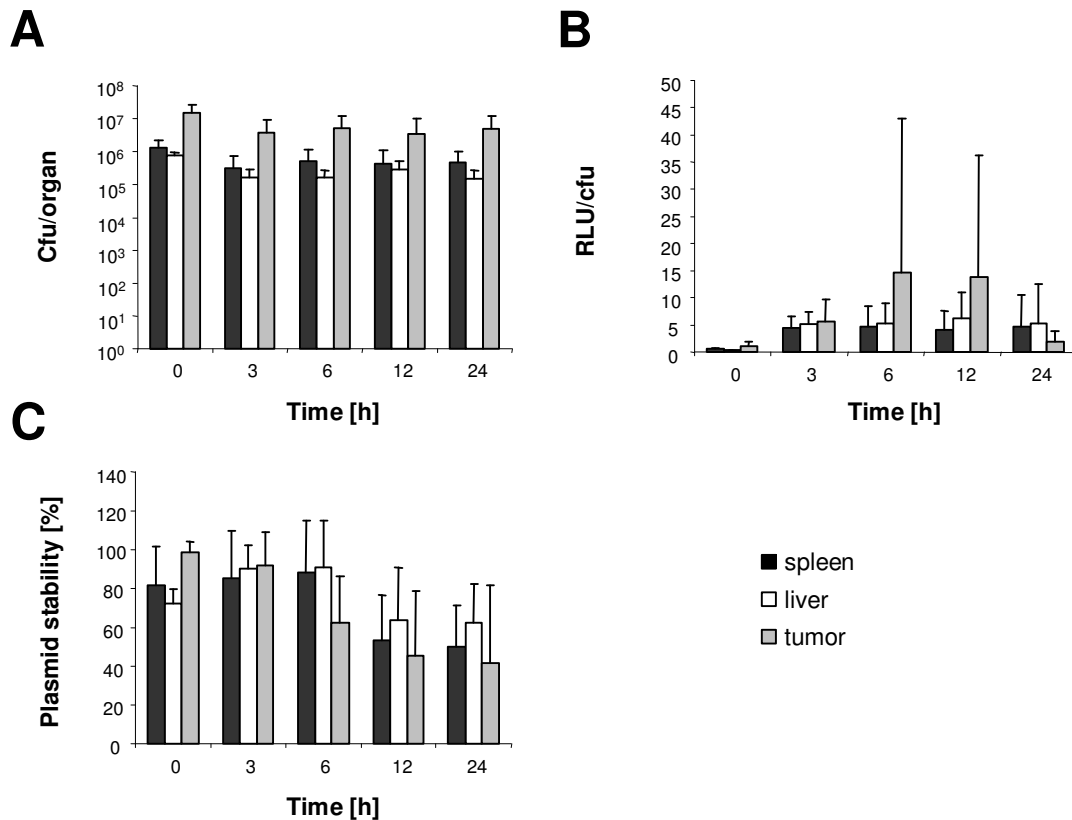


Fig. 3.27: Time course of plasmid amplification after injection of L-arabinose into tumor-bearing BALB/c mice. 3 d p.i. of 5×10^5 SL7207 carrying pAEN59, groups of mice were injected intraperitoneally with 120 mg of L-arabinose. (A) Colonization of host tissue as determined from plating of tissue homogenates at consecutive time points. (B) Kinetics of bacterial luciferase expression as indicator for L-arabinose induced plasmid amplification. Luciferase activity was determined in tissue lysates and calculated as relative light units (RLU) per CFU. (C) Stability of pAEN59 in recombinant SL7207 during the course of induction. Graphs summarize two independent experiments with three mice for each data point (mean + s.d.).

To see how the transient accumulation of L-arabinose in blood influences the induction of plasmid amplification, luciferase activity had to be determined at consecutive time points during the course of induction. Therefore, SL7207 carrying the amplifiable P_{bla} -luciferase expression plasmid pAEN59 were injected intravenously into mice bearing a subcutaneous CT26 tumor of 5-8 mm in diameter. Three days later, 120 mg L-arabinose were injected intraperitoneally and colonization, bacterial luciferase expression and plasmid stability

were determined in tumor, liver and spleen. At least a 10-fold enrichment of bacteria in tumors was observed (Fig. 3.27 A). The bacterial accumulation in tumors was rather variable leading to high standard deviations during measurement of the time course of luciferase activity per CFU (Fig. 3.27 B). Nevertheless, it is obvious that the maximal plasmid copy number was reached within 6–12 h after administration of L-arabinose. Plasmid amplification seemed to result in partial plasmid loss, since the plasmid stability decreased during the course of induction (Fig. 3.27 C).

Expression of genes under control of P_{BAD} is short-lived *in vivo*, since the inducer only transiently accumulates in the tissues (Loessner et al. 2007). However, a repeated induction of plasmid amplification and protein expression by L-arabinose should be possible. To test this hypothesis, tumor-bearing mice were infected intravenously with SL7207 carrying the amplifiable P_{BAD} -luciferase expression plasmid pAEN64 (pSC101 replicon) and repeatedly induced i.p. with 120 mg L-arabinose (Fig. 3.28). Twelve hours after each induction time point, colonization, bacterial luciferase expression and plasmid stability were determined in tumor, liver and spleen.

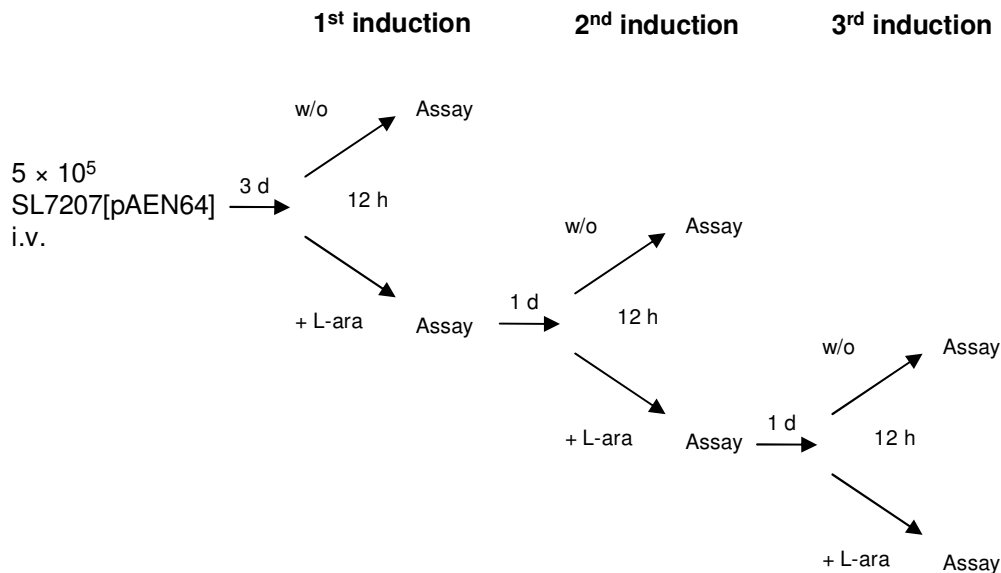


Fig. 3.28: Scheme of repeated induction of plasmid amplification and protein expression by L-arabinose in recombinant *Salmonella in vivo*. Tumor-bearing BALB/c mice were infected i.v. with SL7207[pAEN64]. 3 d p.i., groups of mice were injected i.p. with 0 mg (w/o) or 120 mg L-arabinose and analyzed for tissue colonization, luciferase expression and plasmid stability 12 h later. Another group of mice that received L-arabinose was again injected i.p. with 0 mg (w/o) or 120 mg L-arabinose 36 h after the 1st induction and assayed 12 h later. This procedure was repeated 36 h after the 2nd induction.

Again, recombinant SL7207 preferentially colonized tumors (Fig. 3.29 A) compared to spleen (Fig. 3.29 B) and liver (data not shown). Repeated induction of plasmid amplification and protein expression resulted in plasmid loss and accordingly declining CFU, since displayed values were determined from plating with antibiotic selection. At each induction time point, administration of L-arabinose resulted in higher luciferase expression in bacteria from tumor (Fig. 3.29 A), spleen (Fig. 3.29 B) and liver (data not shown). The induction factor appeared higher in tumor than in spleen; a phenomenon that had been described and can be attributed to the extracellular location of *Salmonella* within tumors (Loessner et al. 2007). Interestingly, the luciferase activity per cfu increased from the 1st to the 2nd induction. This might be due to an additional increase of the plasmid copy number in bacteria, which were not completely induced before. Alternatively, plasmid amplification and protein expression were activated in a portion of bacteria that was not induced at the first administration. Subsaturating inducer concentrations inside tissues could result in gene activation in one but not the other bacterium, a phenomenon called “all-or-nothing” induction of the P_{BAD} promoter (Siegele and Hu 1997).

Similar observations were made when this experiment was performed with SL7207 carrying amplifiable P_{bla} -luciferase expression plasmid pAEN59 or the non-amplifiable P_{BAD} -luciferase expression plasmid pAEN110 (data not shown). In concordance with the *in vitro* data (Fig. 3.24), a lower luciferase activity was determined in both cases compared to SL7207 carrying pAEN64 (data not shown). In summary, it is possible to repeatedly induce plasmid amplification and protein expression with L-arabinose after bacterial colonization of the mammalian host.

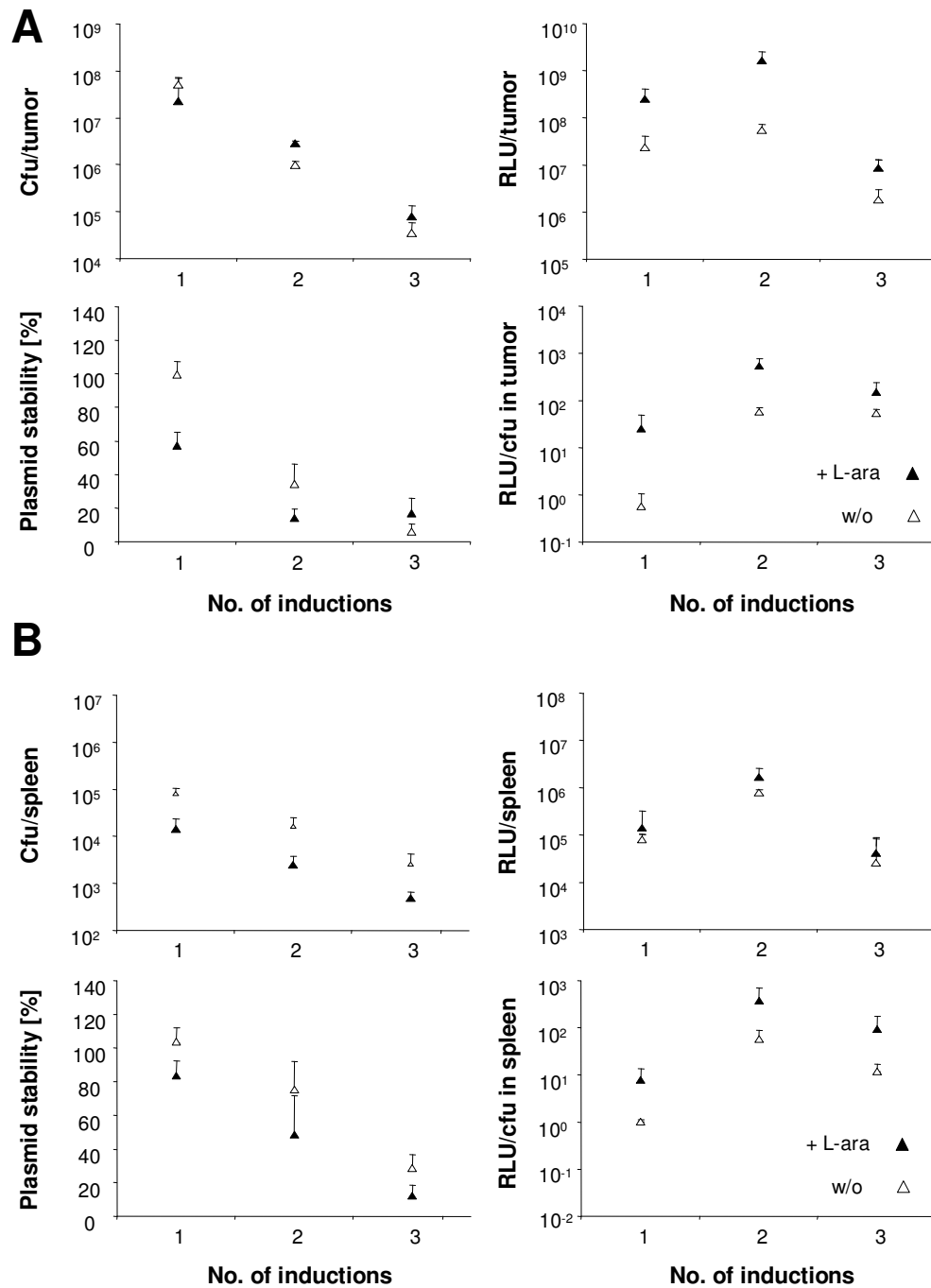


Fig. 3.29: Repeated induction of simultaneous plasmid amplification and protein expression by L-arabinose in tumor-bearing BALB/c mice. Mice were infected with SL7207[pAEN64] and repeatedly induced by L-arabinose as described in Fig. 3.28. 12 h after each induction, colonization, bacterial luciferase expression and plasmid stability was determined in tumor (A) and spleen (B). Values represent the average for three mice per data point (mean + s.d.).

4 Discussion

Over the years, *Salmonella* vaccine strains expressing heterologous antigens or delivering expression plasmids have been used to raise immune responses against a broad range of pathogens as well as to induce antitumor immunity in animal models and to some extent also in humans and non-human primates (reviewed by Garmory et al. 2002 and Loessner and Weiss 2004). One of the conclusions of such trials was that it is of utmost importance to express the heterologous antigens at sufficient levels. This requires that the eukaryotic or prokaryotic expression plasmids are stably retained in the bacterial carrier. Therefore, for the construction of efficient vaccine strains, it is critical to reduce the interference of antigen expression or plasmid load with the viability and fitness of the bacterial carrier. In case of mucosal application, the vaccine strain must also retain its potential to breach the gastrointestinal barrier to reach inductive sites of the immune system (Hansen-Wester and Hensel 2001).

4.1 Establishment of an *in vivo* inducible plasmid amplification system

In the first part of this work, an *in vivo* inducible plasmid amplification system was developed to reduce the metabolic burden of the vaccine carrier especially in the early phase of host colonization. To construct such vaccine strains with a minimal heterologous load, the plasmids contained the replicon of the F plasmid. For inducible plasmid amplification, this was combined with a second RK2-derived origin of replication, *oriV*. In addition, the RK2-derived replication protein TrfA that is responsible for induction of replication via *oriV* was fused to particular *in vivo* inducible promoters and then chromosomally integrated. Such recombinant strains were used to amplify a GFP_{ova} expression plasmid.

As promoters, three previously identified inducible promoters were employed that were known to be activated during intracellular replication of *Salmonella* within professional APC (Rollenhagen et al. 2004). Chromosomally integrated constructs of the reporter gene GFP driven by the promoters P_{sifA}, P_{stm1630} and P_{phoN} confirmed their function in the vaccine strain *S. typhimurium* SL7207. The three chromosomal constructs responded to defined *in vitro* inducing conditions. Under these conditions P_{sifA} performed best as it showed the highest up-regulation combined with low background. Similar findings had been made

previously in *S. typhimurium* strain SL1344 using a pBR322-derived *gfp-ova* reporter plasmid. In this setting, P_{sifA} exhibited a twofold higher *in vivo* expression level compared to $P_{stm1630}$ and P_{phoN} (Rollenhagen et al. 2004).

The initiation protein TrfA mediates assembly of the replication complex at *oriV* in a monomeric form (Toukdarian et al. 1996). Once a plasmid in the bacterial cell is at or above its characteristic copy number, dimeric TrfA takes over the copy number control by coupling *oriV*-bound TrfA monomers (Toukdarian and Helinski 1998). This “handcuffed” complex prevents DNA replication by making it inaccessible for other components of the replication machinery. In the present study, a copy-up *trfA* gene derived from pETcoco-1 (Wild and Szybalski 2004) was used. It was found to have reduced ability to form coupled complexes (Blasina et al. 1996). This might explain why the expression plasmids could be amplified *in vitro* up to 50 copies per bacterial cell when the promoters P_{sifA} and P_{phoN} were used to drive *trfA* transcription.

In infected macrophages and in spleen cells from infected mice, the three promoters showed similar levels of GFP_ova expression as indicator for successful plasmid amplification. As an explanation for the discrepancy between bacterial culture and presence in host cells, bacterial subpopulations with low GFP expression levels might escape detection due to interfering host-cell autofluorescence. Thus, many bacteria containing $P_{stm1630}$ -constructs might not be analyzed. Alternatively, environmental stimuli imposed by the phagosomal compartment of host cells might have a different impact on promoter activity than induction medium. Nevertheless, P_{sifA} had clearly the most attractive induction properties for an *in vivo* inducible plasmid amplification system; i.e. low *in vitro* background and high *in vivo* activity.

As a second step, enhanced antigen expression levels and lower background was achieved by using P_{sifA} for simultaneous regulation of plasmid amplification and reporter gene expression. Interestingly, the strong transcription of GFP_ova observed here did not interfere with amplified replication of the expression plasmid. Transcription from strong promoters had been shown before to interfere with plasmid replication (Stueber and Bujard 1982).

When the F plasmid replicon was exchanged with the alternative low-copy number replicons pSC101 and p15A, GFP_{ova} expression plasmids could be amplified from 5-10 to 100 copies. The twofold higher maximum copy numbers of the pSC101/p15A containing vector compared to the *oriS* containing vector was unexpected. Theoretically, the copy number driven by TrfA and *oriV* should reach a maximum independent of the vector base. In addition, the plasmids were propagated in the same recombinant strain with chromosomally integrated P_{sifA}-*trfA*. Thus, the same amount of the initiator protein should have been available for amplification of all the plasmids. Therefore, it is likely that the different plasmid backbone or the reduced plasmid size lead to a higher characteristic copy number under inducing conditions.

In this context, another unexpected finding was that no significant plasmid amplification was observed in induction medium when strain P_{sifA}-*trfA* carried an expression plasmid based on the low-copy number replicon pMB1. This effect might be attributed to interfering GFP_{ova} transcription in this combination, although antigen expression was apparently reduced in this case. An alternative explanation for this phenomenon might be that replication from origins of replications like pMB1 interferes with *oriV*. From these data, two replicons, either of the F plasmid or pSC101, were selected for further *in vivo* application.

Recombinant strains transformed with amplifiable plasmids based on the F plasmid and pSC101 replicon were therefore tested in colonization studies and compared with control strains. The colonization capabilities of such strains differed depending on their ability to amplify the P_{sifA}-*gfp_{ova}* expression plasmid. Whereas SL7207 carrying only the amplifiable plasmid showed no colonization defect and plasmids were stably retained for more than 7 days after oral immunization, SL7207 bearing amplifying P_{sifA}-*trfA* exhibited plasmid loss and impaired tissue replication. Lower bacterial numbers were observed with both recombinant strains compared to parental strain SL7207 already 1 day p.i.. This indicates either impaired invasion efficiency or impaired survival/replication in host cells. The latter could be due to high protein expression induced by P_{sifA} once the bacteria reside within host cells.

Non-invasive *in vivo* imaging pinpointed at an explanation for the inferior performance of bacteria carrying amplifiable plasmids. Activation of P_{sifA} and plasmid amplification takes

place already in the intestine and hence precedes penetration of the intestinal barrier. This is consistent with a study describing the expression from SPI-2 promoters P_{sseA} , P_{spiC} and P_{ssaG} in the gut within 15 min after application (Brown et al. 2005). The early activation of P_{sifA} obviously results in high antigen expression and contributes very likely to the impaired invasion efficiency observed in the colonization studies.

As one would predict, the colonization defect is enhanced in case of the plasmid containing the pSC101 replicon. This plasmid is lost faster than the *oriS*-containing plasmid in strain P_{sifA} -*trfA*, which is most likely a result of the higher amplification.

It has previously been observed that fluorescent proteins like GFP can lower the fitness of bacterial pathogens (Knodler et al. 2005). Since the same effects were observed using LLO₄₉₋₃₆₁ instead of GFP, the choice of antigen seems to have no impact on the colonization performance. Rather the nature of the plasmid components appears decisive. Consistently, chromosomal insertion of the promoter-*trfA* fusion did not detectably influence the colonization capability of the carrier strain.

Amplified plasmids are retained in carrier bacteria at high percentages for at least three days despite of the metabolic burden imposed. Thus, high antigen expression should be encountered during this time. To relate high antigen expression with the induction of a specific T cell response, a chimeric mouse model was used in which transgenic T cells recognizing the epitope of GFP_{ova} were adoptively transferred into syngeneic, non-transgenic mice (Pape et al. 1997). Oral administration of 1×10^9 SL7207:: P_{sifA} -*trfA* harboring a P_{sifA} -*gfp*_{ova} expression plasmid resulted in a higher stimulation of T cell proliferation compared to the control parental strain. However, a stronger antigen-specific T cell response was observed with intravenous immunization using 5×10^5 cfu of the same strain. Therefore, this route of administration was considered more appropriate to assess the effect of plasmid amplification on the generation of CD4⁺ T cell responses.

Consistently, stronger immune responses could be observed when the recombinant strains were able to amplify the expression plasmids *in vivo*. Obviously, high antigen expression due to plasmid amplification in SL7207:: P_{sifA} -*trfA* compensates for the lower stability of such plasmids. On the other hand, twofold higher amplification of the pSC101 containing plasmid did not lead to stronger T helper cell responses in comparison to the F factor-

containing plasmid. This suggests that prolonged antigen expression due to higher stability of the vector system counterbalances the higher antigen expression in this case. Possibly, a specific optimum of *in vivo* expression levels for every antigen might exist depending on its influence on colonization, stability and immunogenicity.

The superior efficacy of the plasmid amplification system in the generation of CD4⁺ T cell responses remains to be confirmed in an oral immunization scheme. This could be achieved by administering higher doses of recombinant Salmonella. Indeed, 1×10^9 cfu is a rather low dose compared to what has been used in other studies with *S. typhimurium* vaccine vectors ($4\text{--}5 \times 10^9$ cfu) (McKelvie et al. 2004, Hussein and Hensel 2005). In addition, Hussein and Hensel observed that intracellular antigen expression under control of the SPI-2 promoter P_{sseA} was less efficient in strain SL7207 compared to an attenuated strain deficient in the SPI-2 translocation system. Using the *aroA* attenuated carrier strain, regulated Ova expression on a low-copy number plasmid did not result in a significant stimulation of Ova-specific T cell proliferation (Hussein and Hensel 2005). Future work should therefore be directed to the comparison of different attenuated strains of *S. typhimurium* harboring the novel plasmid amplification system. The use of carriers with other attenuations might enhance antigen expression to cognate T cells. On this score, strategies for secretion (Hess et al. 1996) or surface display (Kramer et al. 2003) of the antigen instead of cytoplasmic expression might be advantageous.

An alternative strategy to increase antigen presentation via MHC class II and to induce CD4⁺ T cell responses was pursued in this work. Chromosomal integration of the P_{BAD} -gene *E* lysis cassette next to the P_{sifA} -*trfA* fragment generated a vaccine strain, which allows induction of programmed lysis upon application of L-arabinose. In addition, plasmid amplification was mediated *in vitro*. It remains to be established, whether *in vivo* lysis of the carrier in the phagosomal compartment of APC augments MHC class II-dependent immune responses.

The suitability of the *in vivo* inducible plasmid amplification system for the generation of cytotoxic T cells was evaluated using the model antigen LLO. Independent of the route of immunization, the recombinant Salmonella strains failed to elicit *in vivo* cytotoxicity. This strongly suggests a detrimental effect of the intracellular antigen localization, which can not be compensated by high antigen amounts expressed in the plasmid amplifying strains.

Thus, in order to raise protective CD8⁺ T cell responses, the vaccine strain needs to be improved regarding the feeding of antigen into the MHC class I antigen processing and presentation pathway. In this respect, the use of SPI-2 type III effector proteins to act as carrier molecules for heterologous antigens seems very attractive. Exemplarily, fusion of the model antigen p60 of *L. monocytogenes* to SPI-2 effectors SifA and SspH2 under control of their native promoters and expression from medium-copy number plasmids led to translocation of chimeric p60 proteins into Salmonella-infected APC and in turn to CD8⁺ T cell priming (Panthel et al. 2005). Interestingly, mice immunized with Salmonella expressing chimeric SifA/p60 proteins from low-copy number vectors did not mount measurable immune responses in this study. Thus, the application of *in vivo* inducible plasmid amplification under control of P_{sifA} might be advantageous in this setting. Hence, plasmid amplification and heterologous protein expression would start at the same time as expression of the cognate TTSS since both are specifically controlled via the same environmental conditions. However, the nature of the attenuating mutation in the Salmonella carrier strain has to be considered also for this improvement strategy. Notably, a reduced expression of SPI-2 fusion constructs was observed in a *S. typhimurium aroA* strain in comparison to strains deficient in *htrA*, *purD*, *galE* or *htrA/purD* (Husseiny et al. 2007).

Taken together, this is the first study that demonstrates the *in vivo* inducible amplification of expression plasmids in a Salmonella vaccine strain. Simultaneous regulation of plasmid amplification and antigen expression for Salmonella-mediated protein delivery has several advantages to *in vivo* regulated antigen expression alone. First, expression of the heterologous antigen is presumably minimal during fermentation, eliminating any metabolic burden during vaccine manufacture. The transformants carrying the expression plasmids are stable per se abrogating the need for antibiotics in the growth medium during production. Second, dual control reduces the background antigen expression. This might also allow the expression of heterologous antigens that may be deleterious to Salmonella carrier strains in conventional expression systems. Thus, this approach appears to be very promising for heterologous protein delivery when supplemented with appropriate antigen presentation strategies and in combination with the right attenuation of the carrier. Future work might also investigate the potential of other intracellular activated promoters.

Moreover, the *in vivo* inducible plasmid amplification system is particularly suited for the amplification of DNA vaccines carried by the bacterial vector upon infection of immune cells. Within the framework of this thesis, the system was not evaluated in this respect. However, the amplifiable plasmids could be easily modified for this purpose by exchanging P_{sifA} with a eukaryotic promoter.

The induction of uncontrolled plasmid replication, so-called runaway replication, might also be an interesting strategy for Salmonella-mediated DNA vaccination. Runaway replication leads to an exponential increase in the concentration of plasmid DNA until the cells eventually die (Larsen et al. 1984). Such loss of copy number control could be achieved in our system using a combination of two characterized copy-up TrfA mutations. The double mutant TrfA protein has been shown to be defective in coupling replication origins while being able to bind to *oriV*, thus initiating runaway replication (Blasina et al. 1996). It remains to be tested whether chromosomal expression of the double mutant TrfA under control of P_{sifA} results in uncontrolled plasmid amplification and whether this subsequently augments the DNA transfer.

4.2 Remote control of plasmid amplification by L-arabinose

The deliberate induction of plasmid amplification *in vivo* is an alternative to the use of Salmonella-specific *in vivo* inducible promoters. This strategy appears to be especially promising for tumor therapy. It allows temporal control of the carrier in order to increase specificity, e.g. by administration of the inducer after completion of migration of the bacteria into the tumor. As demonstrated in this work, use of the monosaccharide L-arabinose in combination with the corresponding molecular switch P_{BAD} is highly suitable for that purpose. L-arabinose seems to fulfill all criteria of a versatile inducer. It is a biocompatible food component and is suggested for clinical use in other contexts. Furthermore, it is non-toxic and non-immunogenic to the host and readily available at low cost.

The conditional amplification of reporter plasmids under control of L-arabinose was shown to be tightly regulated *in vitro*. Without addition of the inducer, plasmids were present in a characteristic minimal-copy number state (1-5 copies). This stringent control of plasmid amplification is an important property since it leads to high *in vitro* stability. Depending on the respective first origin of replication, the copy number could be regulated to about

80-100 (F factor) or 250 (pSC101 replicon) copies upon induction. Thereby the *trfA* gene is also amplified due to the episomal location of the P_{BAD} -*trfA* cassette. This seems to augment the amplification effect, since chromosomal expression of *trfA* under control of P_{BAD} resulted only in 8-fold amplification.

In contrast, up to 30-fold amplification of low-copy bacterial artificial chromosomes (BACs) was observed in *E. coli* DH10B carrying a chromosomally integrated P_{BAD} -*trfA* cassette (Wild et al. 2002). This difference might likely be due to the different carriers used, i.e. *S. typhimurium* SL7207 vs. *E. coli* DH10B.

The copy number of the reporter plasmids and in concordance GFP or luciferase expression increased over a wide range of inducer concentrations *in vitro* and *in vivo*. L-arabinose not only reaches Salmonella that reside in the phagosomal compartments of infected J774-A1 macrophages but also reaches bacterial niches in tumor, spleen and liver of infected mice. After systemic administration of L-arabinose the sugar only transiently circulates in the blood of mice and is degraded by host enzymes (Seri et al. 1996). Nevertheless, this leads to L-arabinose concentrations high enough to induce a short-lived expression of genes under control of P_{BAD} (Loessner et al. 2007). Interestingly, the level of constitutive luciferase expression as indicator for plasmid amplification is still rather high 24 h after administration of the inducer, although L-arabinose is completely degraded 7 h after injection. This can be explained by low proliferation rates of such bacteria, since the copy number of amplified plasmids will only decrease when the bacteria divide.

Within the course of induction, plasmid amplification apparently leads to plasmid loss *in vivo*. However, unless induced, amplifiable plasmids harboring the pSC101 replicon are stably retained *in vivo* for two weeks. The reason for this higher stability in comparison to amplifiable plasmids harboring the F replicon is unclear. It might be due the reduced plasmid size (12,2 kb vs. 9,2 kb). However, this property as well as the higher copy number after induction render pSC101 based constructs more suitable than the plasmids containing the F replicon.

Dual control of reporter gene expression and plasmid amplification by P_{BAD} as applied for *in vivo* inducible promoters resulted in increased luciferase expression and lower background. This approach is suitable for Salmonella-mediated delivery of genes, which

are toxic to the carrier and/or the host. Even the most tightly regulated promoters display low levels of background expression and such leaky expression is proportional to the copy number of the plasmid. Therefore, it is desirable to keep the gene dosage very low during manufacture of the carrier strain and its migration within the host. It has been shown that expression of *gene E* from a single chromosomally located copy under control of P_{BAD} did not interfere with growth, invasiveness and tumor colonization (this work and Loessner et al. 2007). Such exceptional tight control could be employed e.g. for the targeted delivery of pro-drug converting enzymes, like cytosine desaminase, or cytokines, like $TNF\alpha$, by tumor-colonizing *Salmonella*. This way, strong expression of the therapeutic gene due to amplification of the gene dosage and initiation of transcription could be spatially and temporally regulated. The inducer could be administered after bacteria have accumulated in the tumor. In addition, a higher induction factor has been observed in tumors compared to liver and spleen, which can be attributed to the extracellular location of *Salmonella* within tumors (Loessner et al. 2007). Furthermore, repeated induction of plasmid amplification and protein expression is possible after bacterial colonization of the mammalian host. Thus, appropriate bacterial vectors may provide antitumor activity for a prolonged period of time.

Plasmid amplification and protein expression could repeatedly be induced by L-arabinose in recombinant *Salmonella in vivo*. The reporter activity increased from the 1st to the 2nd induction time point. This might be due to activation of bacteria, which were incompletely or not at all induced before. On this score, a phenomenon called “all-or-nothing” induction of the P_{BAD} promoter has to be reported. Subsaturating inducer concentrations as they occur very likely inside tissues *in vivo* can result in gene activation in one but not the other bacterium (Siegele and Hu 1997). This can be attributed to fluctuating expression of the L-arabinose transporter AraE in an uninduced state of the endogenous arabinose operon of *Salmonella*. Such bacteria that contain sufficient transporters to accumulate L-arabinose induce the synthesis of additional AraE. These transporters catalyze up-take of additional L-arabinose leading to more induction. This autocatalytic induction cycle continues until a fraction of bacteria becomes fully induced whereas the other subpopulation expressing transporter levels below the threshold is not induced.

Moreover, *S. typhimurium* SL7207 contains functional *araBAD* genes encoding L-arabinose degrading enzymes. Degradation leads to depletion of the sugar and

progressive loss of induction at low L-arabinose concentrations. To circumvent the “all-or-nothing” induction of the P_{BAD} expression system, strains should be engineered that are deficient in L-arabinose transport and degradation genes as described (Morgan-Kiss et al. 2002). In such strains, the sugar entered the cells in a concentration dependent manner due to expression of a mutant LacY transporter that performs facilitated diffusion of L-arabinose.

In experiments with tumor-bearing mice, recombinant *S. typhimurium* strain SL7207 showed a preferential colonization of tumors compared to liver and spleen after intravenous administration. A 10-100 fold enrichment of bacteria in tumor was observed, which is probably not sufficient for targeted tumor therapy. In contrast, higher accumulation ratios (1000:1 – 10000:1) have been observed with other *S. typhimurium* strains in mice (Clairmont et al. 2000, Rosenberg et al. 2002). Therefore, regulated reporter gene expression and plasmid amplification under control of P_{BAD} should also be evaluated in Salmonella strains with better tumor-targeting properties in the future. In this context, one might also employ other remotely controllable promoters for the construction of multifunctional tumor-targeting bacteria, which can be manipulated in several ways *in vivo*.

As an alternative to tumor therapy, remote control of plasmid amplification by L-arabinose might also be used for Salmonella-mediated delivery of heterologous antigens or DNA. This would allow bacteria to infect APC unhampered by expression of antigens and/or by high-copy number of plasmids, both tightly regulated by P_{BAD} . In this setting, the time of induction could be synchronized with the course of the Salmonella infection, i.e. after strong accumulation in lymphoid tissues. Importantly, orally administered L-arabinose has been shown to induce protein expression in Salmonella colonizing Peyer’s Patches (H. Löbner, unpublished observation). Thus, it might be possible to develop an orally administered Salmonella vaccine, which is activated by orally administered L-arabinose.

4.3 Concluding remarks

Taken together, this work describes for the first time a system for *in vivo* inducible plasmid amplification in Salmonella. The system is designed to maintain low plasmid levels until activation of *in vivo* inducible promoters in APC of the host or administration of the external inducer L-arabinose. Both approaches for regulation of the system offer tight control, strong induction and a variety of applications. However, a number of possibilities

for improvement arise from the present work. For instance, the rational design of the carrier strain for vaccination or tumor therapy will be of high interest. Similarly, the system can be extended to other remotely controllable and *Salmonella*-specific promoters, e.g. newly identified tumor-specific promoters. Thus, it should lead to the optimization of *Salmonella*-mediated delivery of prophylactic and therapeutic molecules. This study represents an important step in this direction.

5 Summary

Live attenuated *Salmonella* are highly potential vectors for the delivery of proteins and DNA with applications in vaccination and tumor therapy. Conventional expression systems are based on maintenance of the expression plasmid at multi-copy numbers representing a high metabolic burden for the bacteria that results in a decreased performance of the vector. In this work, a system for inducible amplification of expression plasmids was established in *Salmonella enterica* serovar Typhimurium vaccine strain SL7207. Amplifiable plasmids were constructed that contain two replication origins. The first replicon ensures the minimal-copy number state under non-inducing conditions. A second medium-copy replicon derived from plasmid RK2 is responsible for conditional amplification of the expression plasmids. Activation of the second replication origin depends on the replication protein TrfA. Accordingly, expression of *trfA* was placed under control of a *Salmonella*-specific *in vivo* inducible promoter or a promoter that can be deliberately activated by a low molecular weight inducer.

As *in vivo* inducible promoters, P_{phoN} , $P_{stm1630}$ and P_{sifA} were tested in this work. Such promoters are claimed to regulate genes that are expressed in the vacuole of the host cell. P_{sifA} was found to be most promising. It was silent under non-inducing conditions and exhibited strong amplification of the plasmid copy number up to 50-100 copies. Consequently, enhanced expression of plasmid-encoded antigen was observed *in vitro* in induction medium and upon infection of macrophages. Similar results were obtained after infection of mice. Expression could be further increased by controlling transcription of the antigen via P_{sifA} at the same time.

However, non-invasive *in vivo* imaging demonstrated that P_{sifA} is activated already early in the intestine. This obviously impairs the invasion efficiency of orally administered plasmid amplifying strains. Nevertheless, after intravenous immunization, plasmid amplification resulted in substantial ovalbumin-specific CD4⁺ T cell responses. Induction of CD8⁺ T cells could not clearly be demonstrated under these conditions when listeriolysin O was used as antigen. This was attributed to the antigen localization in the bacterial cell and suggests further experiments with secreted antigens. In addition, *Salmonella*-mediated DNA vaccination should be tested using this newly established *in vivo* plasmid amplification system.

In the second part of this work, plasmid amplification could be induced at will by employing the promoter P_{BAD} and addition of L-arabinose to bacterial cultures and when the bacteria resided within macrophages. More importantly, remote control of amplification was also possible in mice. Salmonella were used to colonize tumors of tumor-bearing mice as well as liver and spleen. The tight control of P_{BAD} led to low background activity and exquisite *in vitro* and *in vivo* stability of amplifiable plasmids in the absence of inducer. Administration of L-arabinose resulted in high reporter activity especially when plasmid amplification and reporter expression were simultaneously driven by P_{BAD} . In addition, repeated induction of reporter gene expression could be demonstrated *in vivo*.

In tumor-bearing mice, strain SL7207 harboring amplifiable plasmids exhibited tumor accumulation ratios of 10-100:1 in comparison to normal tissues. An application of this system in bacterial strains with improved tumor-targeting properties should render it a powerful tool for tumor therapy. Furthermore, the deliberate induction of plasmid amplification could be employed for vaccination. In summary, inducible plasmid amplification should contribute to the optimization of Salmonella-mediated delivery of prophylactic and therapeutic molecules and should add to the tools that are available for clinical use of such bacteria.

6 Abbreviations

ADCC	antibody-dependent cell-mediated cytotoxicity
AIDA	adhesion involved in diffuse adherence
AmpR	ampicillin resistance gene
APC	antigen-presenting cells
asd	aspartate β -semialdehyde dehydrogenase
BAC	bacterial artificial chromosome
BCG	bacille Calmette-Guerin
BHI	brain heart infusion
bp	basepairs
CD	cytosine desaminase
CFSE	carboxy fluorescein succinimidyl ester
cfu	colony forming units
CmR	chloramphenicol resistance gene
CMV	cytomegalovirus
const.	constitutive
CpG	cytosine phosphate guanosine
CTL	cytotoxic T lymphocyte
DAP	diaminopimelic acid
DC	dendritic cells
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetate
e.g.	for example
FAE	follicle-associated epithelium
FC	fluorocytosine
FCS	fetal calf serum
FDA	Food and Drug Administration
FU	fluorouracil
HBV	hepatitis B virus
HIV	human immunodeficiency virus
HPV	human papillomavirus
i.e.	that is
IFN	interferon

Ig	immunoglobulin
IL	interleukin
IM	induction medium
IMDM	Iscoe's modified Dulbecco's Eagle's medium
i.p.	intraperitoneal
ISCOM	immunostimulating complex
i.v.	intravenous
IVI	<i>in vivo</i> inducible
KnR	kanamycin resistance gene
LB	Luria Bertani
LLO	listeriolysin O
LPS	lipopolysaccharides
M cell	microfold cell
MACS	magnetic cell sorting
MALT	mucosa associated lymphoid tissue
MDPR	methylpurine-2'deoxyriboside
MFI	median fluorescence intensity
MHC	major histocompatibility complex
MLN	mesenteric lymph node
MOI	multiplicity of infection
Mp	macrophage
MVA	modified vaccinia virus Ankara
OD	optical density
ori	origin of replication
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethyleneglycol
p.i.	post infection
PI	propidium iodide
PMN	polymorphonuclear cell
PP	Peyer's Patches
RLU	relative light units
Rpm	rotations per minute
RT	room temperature

SARS	severe acute respiratory syndrome
s.c.	subcutaneous
SCV	Salmonella-containing vacuole
s.d.	standard deviation
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
SED	subepithelial dome
SPI	Salmonella pathogenicity island
Spp.	Subspecies
TAE	tris-acetic acid EDTA
TE	trypsin-ETDA
Th cell	T-helper cell
TLR	toll-like receptor
Tn	transposon
TNF	tumor necrosis factor
TO	thiazole orange
TTSS	type III secretion system
u	unit
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume

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Publikationen

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